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RESPOSTAS FISIOLÓGICAS E BIOQUÍMICAS DE GENÓTIPOS DE CACAU À INDUÇÃO DE RESISTÊNCIA A VASSOURA-DE-BRUXA POR MEIO DAS INTERAÇÕES ENTRE ENXERTO E PORTA-ENXERTOS E DO USO DE ELICITORES QUÍMICOS

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ILHÉUS – BAHIA – BRASIL
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DEDICATÓRIA

Aos meus pais,

minha esposa Simone, meus filhos Bruno e Enzo,

meu irmão Erivaldo e minhas irmãs Ana Maria e Ediela

aos meus amigos

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RESUMO

RIBEIRO, Miguel Antonio Quinteiro, Universidade Estadual de Santa Cruz, Ilhéus, fevereiro de 2015. **Respostas fisiológicas e bioquímicas de genótipos de cacau à indução de resistência a vassoura-de-bruxa por meio das interações entre enxerto e porta-enxertos e do uso de elicitores químicos.** Orientador: Alex-Alan Furtado de Almeida. Co-orientador: Carlos Priminho Pirovani.

O cacau (*Theobroma cacao* L.) é um dos mais importantes cultivos tropicais perenes no mundo. No Brasil, a diminuição da produção, nos últimos 25 anos, foi ocasionada principalmente pela entrada e disseminação da vassoura-de-bruxa (VB) (*Moniliophthora perniciosa* Aime & Phillips-Mora) na região cacaueira da Bahia, Brasil, principal região produtora do país. Esta doença aumenta os custos de produção e diminui a produtividade da lavoura, dois fatores fundamentais para a viabilidade econômica do cultivo. Visando avaliar estratégias de controle da doença, dois experimentos foram conduzidos em condições de casa de vegetação. O experimento (i) teve com objetivo, avaliar as interações entre enxerto e porta-enxertos quanto à resistência a VB, por meio de medições de trocas gasosas e fluorescência da clorofila; determinação da atividade de enzimas envolvidas no metabolismo antioxidativo; e determinação do teor de macro e micronutrientes minerais em nível foliar. Observaram-se nos genótipos enxertados e não exertados, que a infecção por *M. perniciosa* promoveu alterações nas fases bioquímica e fotoquímica da fotossíntese, na atividade das enzimas envolvidas no metabolismo

antioxidativo e no conteúdo de macro micronutrientes minerais. Concluiu-se que as interações entre enxerto e porta-enxerto em *T. cacao* tornaram o enxerto mais tolerante à infecção por *M. perniciosa*. O experimento (ii) teve como objetivo avaliar o efeito de elicitores químicos [glicose, sacarose e ácido salicílico (AS)], em relação à indução de resitencia à VB em dois genótipos de cacau, um tolerante a VB (CCN-51) e um intolerante (SIC-876). As respostas das plantas foram avaliadas em nível foliar por meio da concentração de macro e micronutrientes minerais, medições de trocas gasosas e fluorescência da clorofila. Nos dois genótipos estudados, as plantas tratadas com AS apresentaram índices de doença significativamente menores, em SIC-876, sacarose apresentou resultado semelhante ao do AS. O Mn está associado ao efeito dos elicitores estudados.

Palavras-chave: *Teobroma cacao*, *Moniliophthora perniciosa*, enxertia, resistência à doença.

ABSTRACT

RIBEIRO, Miguel Antonio Quinteiro, Universidade Estadual de Santa Cruz, Ilhéus, fevereiro de 2015. **Physiological and biochemical cacao genotypes responses to witches' broom resistance induction through the interactions between scion and rootstocks and the use of chemical elicitors.** Advisor: Alex-Alan Furtado de Almeida. Co-advisor: Carlos Priminho Pirovani.

Cacao (*Theobroma cacao* L.) is one of the most important perennial crops in the world. In Brazil, the decrease in production in the last 25 years was caused by the arrival and dissemination of witches' broom (WB) (*Moniliophthora perniciosa* Aime & Phillips-Mora) in the cacao region of Bahia, the main producing region of the country. This disease increases costs and reduces crop yields, two key factors for the economic viability of farming. In order to evaluate control strategies of the disease, two experiments were conducted under greenhouse conditions, (i) in order to assess the interactions between scion and rootstocks for resistance to WB, through gas exchange and chlorophyll fluorescence measurements; determining the activity of antioxidant enzymes involved in metabolism; and determining the content of macro and micronutrient contents in leaf level. Were observed in the scion and non exertados genotypes that infection with *M. perniciosa* modified the photochemical and biochemical phase of photosynthesis, the activity of enzymes involved in metabolism and antioxidant content of some macro mineral micronutrients. It was concluded that interactions between scion and rootstock in *T. cacao* become the most tolerant scion to *M. perniciosa*. The experiment (ii) aimed to evaluate the effect of chemical elicitors [glucose, sucrose and salicylic acid (SA)], in relation to the

induction resitencia WB in Two genotypes of cocoa trees, a tolerant BV (CCN-51) and a susceptible (SIC - 876). The responses of plants were evaluated in leaf level through the concentration of macro and micronutrient contents, measurements of gas exchange, chlorophyll fluorescence. In both genotypes, the plants treated with AS had significantly lower disease rates in SIC-876, sucrose showed results similar to AS. The Mn is associated with the effect of studied elicitors.

Key words: *Theobroma cacao*, *Moniliophthora perniciosa*, scion, disease resistance

ÍNDICE

1. INTRODUÇÃO

Theobroma cacao L. (Malvaceae) é uma espécie lenhosa preferencialmente alógama e perene, nativa da América tropical (BARTLEY, 2005), pertencente à família Malvaceae (JUDD et al., 2009). É explorado comercialmente para a produção de amêndoas, destinadas ao preparo de derivados e subprodutos do cacau, sobretudo na sua forma mais popular, o chocolate (ALMEIDA; VALLE, 2007; 2009). O Brasil, um dos principais produtores mundiais de cacau, apresentou previsão de safra para o ano de 2014 de 281 mil toneladas, com a Bahia sendo o principal estado, com produção estimada para esse mesmo ano superior a 179 mil toneladas de cacau, representando mais de 64% do total produzido no país (IBGE, 2014).

Em 1989, foi registrada pela primeira vez na região cacaueira da Bahia, a doença do cacaueiro vassoura-de-bruxa (VB) (PEREIRA et al., 1989), cujo agente causal é o fungo *Moniliophthora perniciosa* (OLIVEIRA; LUZ, 2005). Como a região sudeste da Bahia era formada por um grande maciço de áreas contiguas e variedades, na grande maioria intolerante, a doença se alastrou rapidamente por toda região, provocando queda drástica na produção que decresceu de 400 mil toneladas ano para 100 mil (MIDLEJ; SANTOS, 2007).

Os métodos para o controle da VB do cacaueiro incluem o manejo e a poda fitossanitária, que consiste na retirada das partes infectadas; controle químico e biológico, principalmente por meio de fungos endofíticos (MEJÍA et al., 2008); mas apenas o uso de genótipos resistentes tem apresentado uma solução econômica e ambiental viável. O controle químico com fungicidas de contato não é eficiente, pois ele não protege os tecidos em crescimento ativo, necessitando de muitas pulverizações (PURDY; SCHMIDT, 1996). A utilização de técnicas de enxertia permite acelerar esse processo minimizando os custos envolvidos (LEMOS et al., 2010).

A enxertia é prática mundialmente consagrada na fruticultura, tanto de regiões de clima temperado como de clima tropical, sua utilização permite a reprodução integral do genótipo que apresenta características desejáveis (DICKISON, 2000). A enxertia sobre porta-enxertos apropriados oferece uma série de vantagens em relação ao cultivo normal, como redução de doenças causadas por fungos e aumento da tolerância às baixas temperaturas (ODA, 1995)

O estado de resistência contra doenças pode ser induzido sistemicamente pela utilização de agentes externos (indutores) bióticos ou abióticos, sem qualquer alteração do genoma da planta, ocorrendo de maneira não-específica, por meio da ativação de genes que codificam para diversas respostas de defesa (STADNICK; BUCHENAUER, 2000; HAMMERSCHMIDT et al., 2001). Em estudo com plantas de cacau tratadas com cloreto de potássio, glicose, sacarose e ácido salicílico demonstrou ação efetiva na indução a resistência à VB (VIEIRA; VALLE, 2006).

Objetivou-se, no presente trabalho, avaliar as respostas fisiológicas e bioquímicas de genótipos de *T. cacao* à indução de resistência a VB por meio das interações entre enxerto e porta-enxertos e do uso de elicitores químicos.

2. REVISÃO DE LITERATURA

2.1. *Theobroma cacao* L.

O cacau (*Theobroma cacao* L.) é uma espécie lenhosa, típica de clima tropical, diplóide (2n = 20), preferencialmente alógama e perene. Seu provável centro de origem é o alto Amazonas, hipótese de dispersão geográfica natural ampla aventada por Nava (1953), formalizada por Cuatrecasas (1964) e corroborada por León (1987). É considerado como de origem exclusivamente neotropical com dispersão natural em florestas úmidas, estendendo-se da bacia amazônica até o sul do México (CUATRECASAS, 1964). Caracteriza-se por ser uma árvore de pequeno porte, atingindo até seis metros de altura, de copa globosa, com flores pequenas inseridas no tronco e nos ramos principais, de onde também surgem frutos de tamanho e formato variáveis (LORENZI; MATOS, 2002).

Do seu provável centro de origem, na região do alto Amazonas (CHEESMAN, 1944), o *T. cacao* espalhou-se em duas principais direções, o que resultou nos dois

principais grupos raciais: o Crioulo, cultivado na Venezuela, na Colômbia, no Equador, no norte da América Central e no México; e o Forastero Amazônico, Peru, Equador, Amazonia e Guianas. Um terceiro grupo, denominado *Trinitário*, também é apresentado por alguns autores como originário de cruzamentos entre os grupos Crioulo e Forastero Amazônico (ALMEIDA; VALLE, 2007; 2009). A maior parte (95%) da produção mundial de cacau provém do grupo Forastero Amazônico, sendo este predominante também nas plantações brasileiras. A espécie *T. cacao* foi introduzida na Bahia em 1746 (CHEESMAN, 1944), inicialmente, as variedades de cacau introduzidas foram originadas do baixo amazonas, do grupo Forastero, conhecidos como cacau Comum, Pará e Maranhão, sendo multiplicadas e selecionadas de forma massiva pelos produtores locais por quase dois séculos (MONTEIRO; AHNERT, 2012).

A espécie *T. cacao* é comercialmente explorada para a produção de amêndoas destinadas ao preparo de derivados e subprodutos do cacau, sobretudo na sua forma mais popular, o chocolate, podendo também ser transformado em cosméticos, bebidas finas, geleias, sorvetes e sucos (ALMEIDA; VALLE, 2007). O Brasil, um dos principais produtores mundiais de cacau, apresentou previsão de safra para 2014 de 281 mil toneladas (IBGE, 2014).

O sul da Bahia é uma das principais regiões produtoras de cacau do Brasil, com cerca de 70 municípios (SOUZA; DIAS, 2001). Em 1989, foi registrada pela primeira vez, na região cacaueira da Bahia, a doença do cacaueiro VB (PEREIRA et al., 1989). Atualmente esta doença encontra-se disseminada nas Américas do Sul e Central, e Ilhas do Caribe (PURDY; SCHMIDT, 1996). Na Amazônia Brasileira, com especial referência ao Estado de Rondônia, já foram registradas em algumas fazendas perdas de até 90% da produção (ANDERBRHAN, 1984). A região sul da

Bahia, responsável por 75% da produção nacional de cacau, foi a mais afetada, pois o fungo responsável pela doença da VB encontrou nesta área condições ambientais favoráveis para sua rápida disseminação (PEREIRA et al., 1989). Como a região sudeste da Bahia é formada por um grande maciço de áreas contiguas e variedades, na grande maioria, intolerantes, a doença se alastrou rapidamente por toda região, provocando queda drástica na produção que decresceu de 400 mil toneladas ano para 100 mil (MIDLEJ; SANTOS, 2007). Na Bahia, iniciou-se uma drástica mudança no cenário sócioeconômico e ambiental, tendo em vista o fato de ter sido, por muito tempo, a principal fonte de renda desta região (PEREIRA et al., 1989).

2.2. Vassoura-de-bruxa

Dentre as doenças que afetam a cultura cacaueira, a VB é considerada a doença de maior impacto econômico nos países produtores de cacau (PURDY; SCHMIDT, 1996), e a de maior importância sócioeconômica para a cacauicultura brasileira, principalmente para região sudeste da Bahia (RESENDE et al., 2007). Desde a disseminação da doença, a produção do cacau foi reduzida drasticamente, e tornou o Brasil, tipicamente um país exportador de cacau, em importador (PLOETZ, 2007; RESENDE et al., 2007).

O agente causal da VB do cacaueiro é um fungo fitopatogênico *Moniliophthora perniciosa* (Stahel) (Aime & Phillips-Mora, 2005), pertencente à classe dos basidiomicetos. Este agente patogênico é reconhecido mundialmente como importante patógeno do cacaueiro, pois, provavelmente, co-evoluiu com este, já que ambos são endêmicos da Bacia Amazônica (ANDERBRHAN, 1984).

O *M. perniciosa* um organismo hemibiotrófico, pois apresenta duas fases no seu ciclo de vida - a biotrófica (parasítica) e a necrotrófica (saprofítica). O fungo *M.*

perniciosa atua, principalmente, em tecidos meristemáticos em crescimento, tais como brotos vegetativos, almofadas florais e frutos do cacaueiro, com sintomas característicos, resultantes do desequilíbrio hormonal presente na interação hospedeiro-patógeno (SILVA et al., 2002).

A VB provoca, como sintoma característico, a formação dos brotos hipertrofiados, desenvolvimento excessivo nas regiões terminais da planta, aparecimento de inúmeras ramificações com entrenós curtos e folhas geralmente grandes, curvadas ou retorcidas, aparentando vassouras. No início, o desenvolvimento das vassouras é rápido, porém depois de 5-12 semanas começam a secar (WHEELER, 1987), podendo cair ou permanecer na planta. O ataque do fungo nos ramos ou brotos vegetativos provoca inchaços da parte infectada, acompanhada da proliferação de pequenos brotamentos próximos uns aos outros onde se prendem folhas grandes, curvadas e retorcidas, dando o aspecto de uma 'vassoura'. Nas almofadas florais os sintomas também podem ser caracterizados pela formação de vassouras. A gema reprodutiva é revertida para vegetativa que desenvolve brotações. Os frutos provenientes de flores infectadas apresentam forma modificada (frutos com formato de morango) que morrem prematuramente. Quando infectados ainda jovens (1,0 cm de comprimento) ocorre a paralisação de seu crescimento e são produzidas deformações que lhe dão a forma característica de cenoura, secando e apodrecendo em seguida. Os frutos provenientes de flores normais são susceptíveis entre 8 a 12 semanas após a polinização e sob alto nível de infecção apresentam manchas amarelas ou escuras e duras no seu interior, em ambos os casos, apodrecidos ou endurecidos e petrificados. Nos frutos mais desenvolvidos (8 cm de comprimento) pode aparecer mancha negra, dura e irregular

ficando as amêndoas unidas entre si, portanto, inaproveitáveis para consumo (TOVAR, 1991).

A disseminação do fungo ocorre pela dispersão dos basidiósporos pelo vento, necessitando serem depositados rapidamente sobre os locais (sítios de infecção) do hospedeiro (ROCHA; WHEELER, 1985). O ciclo da doença começa quando os basidiósporos germinam na superfície de plantas de cacau e os tubos germinativos penetram nos tecidos jovens diretamente ou através dos estômatos (ANDERBRHAN, 1984).

A recuperação da lavoura cacaueira tem sido gradativa, e consiste, basicamente, na substituição nas lavouras dos materiais susceptíveis por variedades resistentes e produtivas recomendados pela CEPLAC/CEPEC aos cacauicultores, desenvolvidas em programas de melhoramento genético do cacaueiro. Geralmente, a fonte de resistência desses clones deriva de seleções de cruzamentos envolvendo o Scavina 6 (Sca-6) (PINTO; PIRES, 1998; ANDERBRHAN et al., 1998).

O emprego de variedades resistentes e de alta produtividade é a alternativa mais recomendada para se manejar a VB (PINTO; PIRES, 1998). Essa medida de controle é fundamental, haja vista os controles químico e cultural tem se mostrado onerosos e ineficazes, enquanto que o controle genético se esbarra na baixa variabilidade de genótipos resistentes, e o controle biológico, com a utilização de microorganismos antagônicos a *M. perniciosa*, sugere a possibilidade de recombinação sexual, o que favorece a variabilidade genética da espécie causadora da VB (OLIVEIRA; LUZ, 2005).

Diante do quadro atual da cacauicultura no Brasil, métodos alternativos para utilização no manejo integrado da VB do cacaueiro têm sido objetivados, e incluem a busca de fungicidas naturais (BASTOS, 1989; 2004; SILVA; BASTOS, 2007), a

utilização da técnica de enxertia, substituição da copa de plantas suscetíveis a doenças por genótipos resistentes (SIMÃO, 1998), e a indução de resistência com produtos bióticos ou abióticos (RESENDE et al., 2000; 2002).

2.3. Interação enxerto porta-enxertos

A enxertia constitui-se em prática mundialmente consagrada na fruticultura, sendo usada em larga escala, nas principais espécies frutíferas, tanto de regiões de clima temperado como de clima tropical, sua utilização permite a reprodução integral do genótipo que apresenta características desejáveis. É um processo onde duas plantas são justapostas de forma que se unam anatomicamente e fisiologicamente e cresçam como um indivíduo (DICKISON, 2000). A enxertia, igualmente a outros métodos de propagação vegetativa, permite perpetuar as características genéticas da planta de origem (GEORGE; NISSEN, 1987), além disso, as plantas atingem a fase produtiva mais rapidamente em relação à propagação por sementes (HARTMANN et al., 1990).

Historicamente, a enxertia salvou a vinicultura Européia quando o inseto *Dactylosphera vitifoliae* devastou as variedades de videiras européias ao longo do final do século dezenove e início do século vinte. As variedades foram salvas pela enxertia delas em porta-enxertos resistentes oriundos da America do Norte. Mecanismos de resistência podem ser estudados por meio de enxertia (ANSARI; VAN EMDEN 1989; BYGRAVE; BYGRAVE 1998; JACKSON et al., 1985; LAMBERT; KILEN 1984; YOSHIDA, IWANAGA 1991). Schweizer (1938), Buttery (1961) e Yahampath (1968) mostraram a existência de influência do porta-enxerto no crescimento e na produção do enxerto. Entre as principais influências causadas pelo

porta-enxerto em relação ao enxerto, Combe e Gener (1977) e Ng et al. (1982). Os porta-enxertos de plantas cítricas afetam mais de 20 características hortícolas e patológicas da cultivar copa e seus frutos, sendo seu uso considerado essencial na citricultura (CASTLE et al., 1995).

O efeito do porta-enxerto sobre o enxerto no que diz respeito à fotossíntese, crescimento, periodicidade de frutificação, produção de frutos, translocação de água e fotossintatos, e outros fatores, tem sido estudado e documentado em frutíferas decíduas, uma vez que historiadores acreditam que porta-enxertos têm sido utilizados no cultivo de frutíferas por pelo menos 2.000 anos (WEBSTER, 1995). Embora as plantas envolvidas no processo mantenham a identidade genética, há influência de uma sobre a outra. Consideram-se o efeito ananizante ou revigorante, a precocidade na produção e a resistência a pragas e doenças, no enxerto, como, pelo menos em parte, influência do porta-enxerto (HARTMANN et al., 1990). Em seringueira, a seleção de material a ser enxertado levou a aumento de produtividade e resistência a doenças (MARTINS et al., 2000).

Dois aspectos importantes induzidos por porta-enxertos em macieiras são a precocidade (floração e frutificação) e redução no tamanho da planta (TUSTIN et al., 2001;LAURI et al., 2006). Em alguns porta-enxertos a floração na macieira pode ocorrer ao longo do tronco, logo no segundo ano de crescimento das árvores (SELEZNYOVA et al., 2003). Também foi notada a influência do porta-enxerto sobre o crescimento das árvores e eficiência de produção de frutos em laranjeiras (QUAGGIO et al., 2004).

Vários métodos de enxertia são adotados para espécies diferentes, até mesmo dentro da mesma espécie. Eles diferem em termos de tempo necessário para execussão, tipo e método de fixação (LEE et al., 1998; ODA, 1995). A escolha

do tipo de enxerto dentro de determinadas espécies também pode estar relacionado com as condições climáticas e o vigor do porta-enxerto (LEONARDI; ROMANO, 2004). As plantas formam calos na interface do enxerto, o que permite que a água a flua à partir do porta-enxerto para o enxerto quando o calo desenvolve feixes vasculares (MOORE, 1984). Conexões vasculares insuficientes da copa com o porta-enxerto diminui o fluxo de água (TORII et al., 1992). Quando a absorção de água pelas raízes for suprimida na interface enxerto, condutância estomática e crescimento diminuem (ATKINSON; ELSE, 2001; ODA et al., 2005). Assim, arquitetura hidráulica torna-se de fundamental importância, uma vez que o fluxo de água constante controla muitos processos vegetais, tais como crescimento, nutrição mineral, fotossíntese e transpiração (MARTINEZ-BALLESTA et al., 2010).

2.4. Indução de resistência

A resistência da planta a um determinado patógeno é definida, sob aspecto genético funcional, como sendo a capacidade da planta em atrasar ou evitar a entrada e a subseqüente atividade de um patógeno em seus tecidos, por meio de mecanismos de defesa próprios, inativos ou latentes na planta (STICHER et al., 1997; ATHAYDE SOBRINHO et al., 2005; NOJOSA et al., 2005). Vários agentes podem induzir a produção de “sinais” no tecido vegetal, disparando reações que ativam os mecanismos de defesa das plantas (STICHER et al., 1997).

O conhecimento sobre a indução de resistência em plantas contra patógenos é secular (CHESTER, 1933; VALLAD; GOODMAN, 2004). O estado de resistência contra doenças pode ser induzido sistemicamente pela utilização de agentes

externos (indutores) bióticos ou abióticos, sem qualquer alteração do genoma da planta, ocorrendo de maneira não-específica, por meio da ativação de genes que codificam para diversas respostas de defesa (STADNICK; BUCHENAUER, 2000; HAMMERSCHMIDT et al., 2001).

A resistência induzida (RI) é ativada em plantas quando moléculas do indutor se ligam a moléculas receptoras situadas na membrana plasmática da célula vegetal, desencadeando a ativação de vários mecanismos de defesa (RESENDE et al., 2002 a,b). Dessa maneira, o agente externo pode induzir mudanças drásticas na atividade metabólica oxidativa das células ao redor do sítio de invasão, levando a produção de espécies reativas de oxigênio (Reactive Oxygen Species, ROS), que ativam os genes de defesa e a produção de metabólitos antimicrobianos (MACMILLAN, 2002). Este mecanismo, conhecido como reação hipersensível (RH), é caracterizado por uma rápida morte celular no local da infecção (DURRANT; DONG, 2004; HAMMOND-KOSACK; JONES, 2000).

A RI pode ser dividida em duas categorias, a resistência sistêmica adquirida (SAR) e a resistência sistêmica induzida (ISR) (VAN LOON et al., 1998). Na SAR, a resistência desenvolve-se de forma sistêmica em resposta a um patógeno que causa uma lesão necrótica (HR), por aplicação exógena de ácido salicílico ou de outros compostos, como o acibenzolar-S-metil (ASM) e o ácido 2,6-dicloroisonicotínico (INA). Após o aparecimento das lesões necróticas, observa-se uma resistência localizada, na qual células vizinhas às lesões necróticas são induzidas no reforço das paredes celulares via lignificação, deposição de calose e formações de papilas (STICHER et al., 1997).

Na SAR, a resistência expressa, geralmente, é efetiva contra um amplo espectro de patógenos, e está associada à produção e acúmulo de proteínas

relacionadas à patogênese (PRP's), e a proteínas ricas em hidroxiprolina, como também a síntese de fenilpropanóides, produtos do metabolismo secundário, como fitoalexinas e compostos fenólicos, síntese de ácido salicílico, e ativação de enzimas chaves como as peroxidases (LEE et al., 1995; DURRANT; DONG, 2004; CAVALCANTI et al., 2005). Já na ISR, não há acúmulo de proteínas PRP's, e o agente indutor não é patogênico, geralmente induzida por rizobactérias. A sinalização está associada a jasmonatos e etileno, mas assim como o ácido salicílico potencializam as respostas dependentes de ROS (GLAZEBROOK, 2005). O indutor não provoca sintomas, como necrose no local da infecção, mas induz a planta a se proteger sistemicamente (VAN LOON et al., 1998; PIETERSE et al., 1998).

Dessa maneira, a proteção obtida contra um determinado patógeno pode ser local ou sistêmica, dependendo do intervalo de tempo entre o tratamento inicial (indutor) e a inoculação do patógeno (desafiador). A sua duração pode ser de poucos dias a algumas semanas, ou mesmo durar todo o ciclo de vida da planta (PASCHOLATI ; LEITE, 1995).

As PRP's foram descritas pela primeira vez em 1970 por Van Loon, que observou o acúmulo de proteínas incomuns após infecção de plantas de fumo com o vírus TMV (DURRANT; DONG, 2004), e foram inicialmente definidas como proteínas ácidas, de baixo peso molecular, resistentes a proteases e solúveis em ácidos, localizadas nos espaços intra e intercelulares. As PRP's presentes nos vacúolos exercem efeito de defesa após a descompartimentalização das células, enquanto que as extracelulares atuam diretamente em contato com o patógeno no processo de penetração do tecido (STICHER et al., 1997).

A ativação das defesas das plantas pode ocorrer a partir da elicitação por compostos bióticos presentes em extratos de plantas (STANGARLIN, et al., 1999), preparações de leveduras (STADNICK; BUCHENAUER, 2000), exopolisacarídeos bacterianos (CASTRO; BACH, 2004), rizobactérias promotoras de crescimento (VISWANATHAN; SAMIYAPPAN, 2002), fungos promotores de crescimento (MADI; KATAN, 1998), e ainda raças não virulentas do patógeno (MONOT et al., 2002), além do próprio patógeno inativado pelo calor (BACH et al., 2003). Pode-se ainda utilizar elicitores abióticos, como silício (Si) (CHÉRIF et al., 1994), ácido salicílico (AS) (CIPOLLINI, 2002), ácido jasmônico (AJ) (BALDWIN, 1998; CIPOLLINI, 2002), glicose e sacarose (VIEIRA; VALLE, 2006).

De maneira geral, os eliciadores comumente isolados e identificados pertencem a diferentes classes químicas, como carboidratos, peptídeos, proteínas, lipídeos, glicoproteínas, glicopeptídeos, ácidos graxos, entre outros (NÜRNBERGER; BRUNNER, 2002; WALTERS et al., 2005). Assim, além de produtos químicos e eliciadores específicos, estudos realizados com diversas culturas, incluindo o cacaueiro, tem demonstrado o efeito indutor de vários nutrientes ou micronutrientes no aumento da resistência a doenças, uma vez que atuam como cofatores de enzimas envolvidas na síntese de compostos fenólicos, como no caso do manganês (SILVA et al., 2008). Em estudo com plantas de cacau tratadas com cloreto de potássio, glicose, sacarose e ácido salicílico demonstrou ação efetiva na indução a resistência a VB (VIEIRA; VALLE, 2006).

Fisiologicamente, a utilização de elicitores com objetivo de induzir resistência requer muitas vezes um custo de adequação das plantas, podendo apresentar efeito negativo no seu desenvolvimento e na produção quando as plantas não estão infectadas (HEIL et al., 2000). Toda alocação de recursos para a defesa pode ser

considerado como custo geral, porém, este custo é dividido entre defesas constitutivas e induzíveis (COLEY et al., 1985).

As plantas, de um ponto de vista evolucionário, desenvolveram um sistema de defesa latente, com a finalidade de economizar energia e substrato, que pode ser ativado com a chegada do patógeno, ao contrário da resistência constitutiva, que representa um custo real à planta, uma vez que independente da presença do patógeno, esta investe seus limitados recursos na defesa. De acordo com a hipótese de alocação de recursos, é previsto que, quando os patógenos estão presentes o investimento de defesa deve valer a pena e as plantas induzidas serem beneficiadas (COLEY et al., 1985). Desta maneira, a resistência induzida em condições naturais representará custo apenas na presença do patógeno (HEIL; BALDWIN, 2002), e, mesmo com a chegada deste, há uma compensação pelo atraso temporal na expressão de defesa, alocando recursos para este propósito somente quando necessário (BOSTOCK, 2005).

O custo da indução de resistência é difícil de ser mensurado, pois seria necessário um hospedeiro suscetível no estado inteiramente não induzido (KUHN et al., 2006). Devido à dificuldade de mensurar os custos totais das defesas induzidas, pesquisadores têm determinado apenas os custos de um indutor específico entre plantas induzidas e não induzidas (PURINGTON, 2002). Divergências podem ser geradas quando se comparam espécies ou variedades de plantas (DANN et al., 1998), dessa forma, para se comparar diferentes sistemas e se extrapolar para condições naturais, deve-se considerar a concentração do elicitor, o método de aplicação, a espécie da planta hospedeira e o estádio de crescimento (HEIL et al., 2000).

3. CAPÍTULO I

Interactions between Scion and Rootstock and Resistance to *Theobroma cacao* Witches' Broom: Photosynthetic, Nutritional and Antioxidant Metabolism Responses

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Abstract

Cacao (*Theobroma cacao* L.) is one of the most important perennial crops in the world. In Brazil, the decrease in production in the last 25 years was caused by the entry and dissemination of witches' broom (WB) (*Moniliophthora perniciosa* Aime & Phillips-Mora) in the cacao region of Bahia, the main producing region of the country. This disease increases costs and reduces crop yields. The main objective of this study was to evaluate interactions between scion and rootstocks for WB resistance through gas exchange and chlorophyll fluorescence measurements, determinations of the activity of enzymes involved in the antioxidant metabolism and of macro and micronutrient concentration at the leaf level. The experiment was conducted under greenhouse conditions using two cacao clones as rootstock, a tolerant (*SCA-6*) and a susceptible (*SIC-876*) to WB and *CCN-51* as scion, a reference material due to tolerance to the disease, good productivity and high beans butter concentration. No grafted plants of SCA-6, SIC-876 and CCN-51 were used as controls. Were observed either in the grafted and non-grafted genotypes that *M. perniciosa* infection provoked alterations in the photochemical and biochemical phases of photosynthesis, in activity of enzymes involved in the antioxidant metabolism and in macro and micronutrients concentrations. It was concluded that interactions between scion and rootstock in *T. cacao* made the scion more tolerant to *M. perniciosa* infection. SIC-876 rootstock, considered susceptible to WB, had a positive effect on the performance of CCN-51 graft with respect to tolerance to the disease. The higher tolerance of CCN-51 graft to WB, provided by the SIC-876 rootstock, was mainly due to increased activity of ascorbate and guaiacol peroxidases, enzymes involved in the antioxidant metabolism.

Keywords: cacao, *Moniliophthora perniciosa*, leaf gas exchange, chlorophyll fluorescence emission, oxidative stress, macro and micronutrients.

Introduction

Cacao (*Theobroma cacao* L.) is one of the most important commodity crops in the world. Its cultivation involves nearly two million producers in more than 50 countries [1]. It is mainly exploited for chocolate manufacture, however, can also be used for cosmetics, beverages, jellies, creams and juice production [2]. Brazil, one of the leading cacao producers, showed a crop forecast for 2014 of 281 thousand tons. Bahia, main cacao producer state, participates with an estimated yield of over 179 thousand tons, which represents more than 64% of total country production [3]. However, the cacao crop in Bahia continues in crisis. The main elements identified as responsible for the current situation are the damages caused to the cacao plant, with reflexes in yield, by witches' broom (WB), a disease caused by the basidiomycete *Moniliophthora perniciosa* (Aime & Phillips-Mora). In addition, low prices of the product in the international market caused by the increased production of African countries, as well as, the lack of capital for technology application faced by farmers also contributed to the crises [4,5].

Witches' broom was first detected in Bahia in 1989. The disease is endemic to the Amazon region and is considered one of the most important cacao maladies [6]. Typically, the fungus disseminate by wind, water and propagation material (cuttings and contaminated seedlings) [7]. When control measures are not adopted, it can reduced yield in up to 90% [4,6]. The fungus shows two different morphophysiological phases during its life cycle, a parasitic and a saprophytic phase. In the first, the pathogen requires reproductive or vegetative living tissues of the host

for nutrition; in the saprophytic phase it feeds on dead tissue [4,7]. In young plants, the fungus mainly infects the apical bud, producing many side shoots (brooms) undesirable for plant growth. In addition, swelling of pulvinus, formation of cankers and fall of dry leaves can occur, causing seedling death. In adult plants overbudding of the apical branch with proliferation of lateral buds (brooms) followed by thickening and shoot death occurs. In infected flower cushions brooms, abnormal flowers and altered fruits (strawberry and carrot types) different from those produced in uninfected flowers are characteristic. In pods early yellowing, dark husks with depressed lesions, damages to seeds and pulp and hardened pods are typical symptoms. In more advanced disease stages, and under high humidity conditions, the emergence of sporocarps (mushrooms) on the surface of the infected pod occurs [4,7].

Grafting is an ancient technique used to combine desirable attributes of the rootstock with those of the plant donor of the graft, the scion. Generally, it is by grafting that susceptible plant canopies are replaced with disease-resistant genotypes. The rootstock acts on the scion altering its development, productivity, time of maturity, size, quality, nutrition and resistance to low temperatures and diseases [8]. In *Vitis vinifera*, the rootstock has a substantial influence on vegetative growth, leaf gas exchange and water status of the canopy [9,10,11]. In *Hevea brasiliensis,* Buttery [12] and Yahampath [13] showed the rootstock influence on growth, yield and disease resistance of the graft. Furthermore, Combe and Gener [14] and Ng et al. [15] demonstrated the existence of great intraclonal variability for vigor and yield between rootstock and scion interactions. In *Citrus*, different rootstocks and scion combinations resulted in physiological alterations in water relations and leaf gas exchange [16].

Plants, when subjected to biotic and abiotic stresses, enhance the production of reactive oxygen species (ROS), which, in excess, are highly toxic and result in oxidative stress [17]. Under stable conditions, ROS molecules are removed by various antioxidative defense mechanisms. These protection mechanisms may be enzymatic or non-enzymatic. Enzymatic mechanisms involve increased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and catalase (CAT), among other enzymes [18]. Non-enzymatic mechanisms, through redox processes, involves ascorbic acid (vitamin C), glutathione, proline, α-tocopherol (vitamin E) and flavonoids [19], which will act to control oxidation cascades and protect plants against oxidative damage [20].

Fluorescence emission of chlorophyll, in turn, is a widely used parameter to assess the photosynthetic capacity altered by biotic and, or abiotic stresses. One advantage of this method is that measurements are not destructive (*in vivo*), causing no damage to the plant, and can be performed either in the laboratory or under field conditions. Several studies have examined the relationship between chlorophyll fluorescence and photosynthesis and physiological status of plants [21,22,23]. Chlorophyll fluorescence assesses the photosynthetic efficiency and provides important information on the structural and functional relationship of photosystem 2 reaction centers (PS2) [21,24].

From the hypothesis that tolerance to WB varies in *T. cacao* and that grafting is widely used to obtain more productive and tolerant to WB plants, this study aimed to evaluate the interaction between scion x rootstock for resistance to WB. The variables used to assess the interaction were measurements of gas exchange, chlorophyll fluorescence, enzymes activity of the antioxidant metabolism and macro and micronutrient concentrations at the leaf level.

Materials and Methods

Plant material and growth conditions

The experiment was conducted under greenhouse conditions at the Cacao Research Center (CEPEC), main research unit of the Executive Commission for the Cacao Farming Plan (CEPLAC), located in Ilhéus, Bahia, Brazil (39º13'59" W, 14º45'15" S, 55 m above sea level). Were used seeds, obtained by controlled pollination at the Active Germplasm Bank (AGB) of CEPEC, of contrasting genotypes for tolerance to *M. perniciosa* (SIC-876, SCA-6 and CCN-51). SIC-876 is a self-compatible genotype with midsize plants and medium to high yield. The main characteristic of SIC-876, a natural mutation in the *T. cacao* variety *'Comum'*, is the absence of anthocyanins in cells of young leaf tissue, as well as young and mature pods and seeds, although there are pods that show ocher seeds due to cross pollination [25]. It is highly susceptible to *M. perniciosa* and has moderate resistance to *Phytophthora palmivora*. On the other hand, SCA-6 is considered a wild type genotype, down-sized, self-incompatible, low yielding, with moderate to high resistance to *M. perniciosa*, moderate to *P. palmivora* and susceptible to *M. roreri* [26]. SCA-6 shows young leaves, fruits and seeds slightly red by the presence of anthocyanins [25]. CCN-51 (Collection Castro Naranjal) was developed by Homero Castro in the early 1960s in Ecuador, from the cross of (*ICS 95* x *IMC 67*) x *Oriente 1*. Castro collected Oriente 1 on his first trip to the Valley of Canelos in the Ecuadorian Amazon. CCN-51 is self-compatible and shows high yield and tolerance to diseases [27].

Initially, the seeds were pre-germinated for 48 h in sterile, water-moistened sawdust. Soon after were sown in plastic tubes of 3.0 L, filled with soil and

commercial substrate Plantmax™ (3:1 v/v), and grown under greenhouse conditions for six months, with daily irrigation.

Grafting process

Plant were grafted six months after sowing using the top grafting technique. During the grafting process, the plants were cut 0.10 m above the cotyledonar scar. Half of SIC-876 and SCA-6 plants were grafted with plagiotropic cuttings collected from five-years old mother plants of CCN-51, from CEPEC's AGB. Not grafted seminal plants of SIC-876, SCA-6 and CCN-51 were used as controls.

Plant Inoculation

Sixty days after grafting, control and grafted plants were inoculated with a suspension of 2 x 10^5 basidiospores mL^{-1} of *M. perniciosa*. On the inoculation day, the spores were removed from liquid nitrogen and diluted in glycerol [28]. Only suspensions showing over 80% spores germination were used for inoculation.

Previously, one day before inoculation, the grafted and non-grafted plants were transferred to a humid chamber with temperature around 25°C and relative humidity of 100%. The plants were inoculated with a 20 µL suspension drop deposited on the apical meristem. The control plants received a 0.2% agar-water droplet. After inoculation, all plants remained for 24 h in the humid chamber. Afterwards, were transferred to a climatized greenhouse, where remained for 30 d, irrigated daily by spraying for 20 min at 09:00 and 14:00 h and nebulization at 12:00 h. After this period, the plants were transferred again to a greenhouse under normal environmental conditions.

Disease evaluation

We evaluated the symptoms presented by grafted (plagiotropic axis) and not grafted control plants (orthotropic axis) individually at 30 and 60 d after inoculation. We determine broom types, number of axillary brooms and diameter and height of brooms when the main symptom was terminal broom. For data analysis, two variables were used: the percentage of plants showing any disease symptoms (SINT), that is, terminal, axillary, cotyledonary and dry brooms, as well as stem, hypocotyl, petiole and pulvinus swelling and also canker, multiple shoots or hypertrophy (Fig. 1). The second variable was disease index (ID), calculated by ID= VT + VA (0.05 * CVT) + NVA, where VT = number of terminal brooms, VA = number of axillary brooms, CVT = length of terminal brooms and NVA = number of axillary brooms larger than 0.01 m. SINT was designed as a discrete binary variable, where 0 or 1 represent absence or presence of symptoms, respectively. Data were analyzed considering the presence or absence of symptoms, as measured of disease incidence (SINT) and ID as a measure of disease severity.

Antioxidant metabolism

We collected the second or third mature leaf from the apex of grafted and not grafted plants 48 h after inoculation. Immediately thereafter, the leaves were frozen in liquid nitrogen, stored at -80°C in ultrafreezer and subsequently lyophilized. For determination of enzymatic activity, approximately 20 mg of leaf tissue of each genotype and treatment were macerated in liquid nitrogen. Then phosphate buffer (50 mmol L^{-1}, pH 6.0) was added at a 20:1 ratio (buffer: dry matter). The samples were ultrasonicated (ultrasonic processor Gex 130, 130 W) on ice to total tissue

disruption with 8 s pulses at 10 s intervals and 70% to 80% amplitude. Afterwards, the sample was centrifuged for 5 min at 13000 x g.

Superoxide dismutase (SOD)

SOD activity was determined according to methodology described by Gianopolitis and Ries [29], with some modifications. One activity unit (UA) was determined as the ability of the enzyme to inhibit 50% photoreduction of nitroblue tetrazolium (NBT) to formazan blue. To the crude extract were added the extraction buffer (potassium phosphate 50 mM, pH 6.0), EDTA (1 mM) and methionine (13 mM). The enzymatic activity was started by the addition of riboflavin (1 mM). The initial reading took place after the plate was in the dark for 5 min and the second after the plate was subjected to 15 W fluorescent light for 5 min. Were considered blanks the wells with no vegetal extract. Readings were taken in a microplate spectrophotometer Espectramax Paradigm (Molecular Devices, CA, USA) at 560 nm.

Catalase (CAT)

The determination of CAT activity was performed according to Havir and McHale [30] methodology with modifications. The activity was measured by the rate of H_2O_2 consumption in the reaction. The determination was performed at 30°C, using reaction buffer (sodium phosphate buffer, 50 mM, pH 7.0) with addition of 1 µL of the plant extract. The reaction was initiated by addition of 30 mM H_2O_2 and readings were made by calculating the decay at 240 nm for 300 s against a blank free of plant extract and expressed in mmol g H_2O_2 DM^{-1} min^{-1} using the molar extinction coefficient of 36 M^{-1} cm^{-1}. The readings were done in a microplate spectrophotometer Espectramax Paradigm (Molecular Devices, CA, USA).

Ascorbate peroxidase (APX)

APX activity was determined by procedures described by Nakano and Asada [31] with some modifications. During the reaction, the presence of APX in plant extract decreases H_2O_2 concentration in the medium in accordance with reduction of added ascorbic acid. To the plant extract was added, adequately diluted, the reaction buffer (50 mM potassium phosphate, 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2). The reaction was initiated after addition of ascorbate. The decay was monitored at 290 nm for 300 s with readings every 30 s. The determination of the activity was performed in a microplate spectrophotometer Espectramax Paradigm (Molecular Devices, CA, USA).

Guaiacol peroxidase (GPX)

For the determination of GPX activity 96 microplate wells were prepared containing 140 µL of GPX 2x reaction buffer [40 mmol guaiacol, 0.06% H_2O_2 and sodium phosphate (20 mmol, pH 6.0)], 139 µL phosphate buffer (50 mmol, pH 6.0) and 1.0 µL of enzyme extract. Sample readings were performed in a microplate spectrophotometer (VersaMax, Molecular Devices, CA, USA) at 470 nm. GPX activity was expressed as the increase in guaiacol consumption in µmol s^{-1} g^{-1} DM. Conversion of data from absorbance values at 470 nm min^{-1} g^{-1} DM to guaiacol consumption in mmol h^{-1} g^{-1} DM was done using the equation y = 0.0189 + 0,1284x (R^2 = 0.99) originated from a standard POD-guaiacol curve [32].

Leaf gas exchange

Gas exchange measurements were done 60 d after inoculation using a Li-6400 portable photosynthesis system (Li-Cor Biosciences, Inc. NE, USA) equipped

with a RedBlue 6400-02B artificial light source. The determinations were performed in the second or third mature leaf from the apex of grafted (plagiotropic axis) and not grafted control plants (orthotropic axis) between 08:00 and 12:00 h. Photosynthetically active radiation (*PAR*), CO_2 flux within the chamber and block temperature of the device were kept constant at 800 µmol photons m^{-2} s^{-1}, 380 µmol mol^{-1} and 26°C, respectively. During measurements, the minimum time for reading stabilization was 60 s, and the maximum to save each reading was 120 s. The maximum admitted value of the coefficient of variation to save each reading was 0.3%. Net photosynthetic rates (*A*), transpiration (*E*) per unit leaf area and stomatal conductance to water vapor (*gs*) were estimated by the difference in CO_2 and humidity values before and after passing through the chamber, determined by the infrared gas analyzer of the device at PAR ≥ 400 µmol photons m^{-2} s^{-1}. We also calculated the instantaneous water use efficiency (*A/E*).

Chlorophyll fluorescence

The determinations were performed on the same leaves used for gas exchange measurements between 08:00 and 12:00 h, totaling eight measurements per treatment. The measurements were done with a portable fluorometer (Handy-PEA, Hansatech Instruments Ltd. Norfolk, UK). Part of the median region of selected leaves was adapted to darkness, using suitable clips, for a period of at least 20 min. This procedure reflected incident solar radiation, decreased temperature and oxidized the photosynthetic electron transport system. Immediately after dark adaptation, the fluorometer was coupled to the clip, which, after opening, received a saturating actinic light pulse (3000 µmol m^{-2} s^{-1} and 650 nm wavelength) for 1 s. The fluorescence emission signals were recorded in the acquisition data system of the

equipment. The JIP test parameters [33] were calculated using the Biolyzer software (Bioenergetics of Laboratory, University of Geneva, Switzerland). Among the obtained parameters were only evaluated the initial (F_0), variable (F_v) and maximum (F_m) fluorescences and the maximum quantum yield of PS2 (F_v/F_m).

Mineral macro and micronutrients

Samples were collected also in the second or third mature leaf of grafted plants (plagiotropic axes) and not grafted control (orthotropic axis) plants between 08:00 and 09:00 h at 60 d after inoculation. Immediately after, the samples were stored in labeled paper bags and dried in an oven with forced air at 75°C until constant mass. Subsequently, the dried plant material was ground in a Wiley mill and stored in identified capped glass bottles.

To determine the concentration of mineral micro and macronutrients, the glass bottles containing the dry biomass were placed uncapped back in the oven at the same temperature for 24 h. Soon after, the vials were transferred, in small numbers, to a desiccator containing dried silica gel, where they stayed for a few minutes before the time of weighing. Immediately after, 200 mg of each sample were weighed using an analytical balance (Shimadzu AY220; Shimadzu Co. Tokyo, Japan). Then leaf samples were submitted to nitropercloric digestion for Ca, Mg, Fe, Zn, Cu and Mn determination by atomic absorption spectrophotometry, P by colorimetry using the vitamin C method [34], and K by flame emission photometry. Nitrogen content was determined by the Kjeldahl method, after sulphosalicylic digestion of leaf samples [34]. The results were expressed as mg plant^{-1}.

Statistical analysis

The plants were arranged in a completely randomized design with 10 treatments (SIC-876, SCA-6, CCN-51, CCN-51 grafted in SIC-876 and CCN-51 grafted in SCA-6 with and without inoculation by *M. perniciosa*), four replicates and 25 plants per experimental unit. Analyses of variance (ANOVA) and comparison of means using Tukey's test ($p < 0.05$) were done. Also, mean comparisons for the antioxidant metabolism, leaf gas exchange, chlorophyll fluorescence emission, macro and micronutrients concentrations were performed for plants with or without WB using Student's t test ($p < 0.05$).

Results

Disease evaluation

Results were analyzed considering the proportion of brooms as an incidence variable (SINT) and the disease index (ID) as a severity variable (Fig. 1). The broom incidence in the susceptible material (SIC-876), as expected, was significantly higher than in the other materials studied (Fig. 1). However, CCN-51 grafted onto SIC-876 showed SINT and ID values statistically equal to SCA-6 and CCN-51 of seminal origin. As rootstock, SIC-876 significantly outperformed SCA-6. There was a significant decrease in SINT and ID values in the combination CCN-51 x SIC-876, when compared to CCN-51 grafted on SCA-6. Although the SIC-876 is considered susceptible to WB, this characteristic did not negatively influence the CCN-51 graft. There was no significant difference between seminal CCN-51 (no graft) and the

grafted on SCA-6. The interaction between the susceptible rootstock (SCI-876) and the tolerant graft (CCN-51) was positive. Thus, the results obtained for SINT and ID in CCN-51 grafted on susceptible SCI-876 rootstock show lower disease incidence and severity (Fig.1).

Antioxidant metabolism

SOD activity was significantly ($p < 0.05$) higher in inoculated SCA-6 followed by CCN-51. The other treatments did not show statistical differences (Fig. 2). Similar behavior was observed for the not inoculated treatments. Also, no significant differences between inoculated and not inoculated plants with *M. perniciosa* spores were found, except in SCA-6 and CCN-51 grafted on SCA-6 (Fig. 2).

In addition, significant variation ($p < 0.05$) was observed in CAT activity in the studied treatments. However, there were no significant differences between plants inoculated and not inoculated with *M. perniciosa* (Fig. 3), with exception of CCN-51. However, CCN-51 grafted on SCA-6 showed significantly higher CAT activity than CCN-51 grafted onto SIC-876 on both inoculated and not inoculated plants. SCI-876 showed significantly lower CAT activity on both inoculation treatments (Fig. 3).

In general, no significant variations in APX activity were found among treatments inoculated with *M. perniciosa* (Fig. 4), except for CCN-51, whose activity value was significantly lower ($p < 0.05$). However, among non-inoculated plants, the highest APX activity was observed in SCA-6 (seminal) and CCN-51 grafted onto SCA-6, while for both seminal CCN-51 and SIC-876 the values were significantly lower. APX mean activity value for CCN-51 grafted onto SIC-876 was intermediary. On the other hand, APX activities of all inoculated treatments were significantly higher than the not inoculated counterparts (Fig. 4).

The highest values for GPX activity were found in inoculated SCA-6 (seminal) and CCN-51 grafted on SIC-876 (Fig. 5). The lowest values were found in non-inoculated plants of CCN-51 (seminal) and CCN-51 grafted on both SCA-6 and SIC-876. Furthermore, for most treatments, GPX activity was significantly ($p < 0.05$) lower in non-inoculated plants, except for SIC-876 (seminal) and CCN-51 grafted onto SCA-6, whose GPX activity values did not differ between inoculated and non-inoculated plants.

Photosynthetic responses

Overall, we found that the presence of WB led to a significant decrease in net photosynthetic rates (*A*) of grafted and ungrafted plants, except for CCN-51 grafted on SIC-876 (Fig. 6). In general, infected plants showed a trend of lower *A*; however, the carbon assimilation rate was significantly lower on CCN-51 ($p < 0.05$). On the other hand, non-infected plants showed higher *A* rates than inoculated plants, except for CCN-51 grafted on SIC-876. In addition, significant differences in *A* were found between inoculated and non-inoculated plants in all treatments except for the combination CCN-51 grafted on SIC-876.

Form most treatments gs was significantly higher ($p < 0.05$) in plants without inoculation except for seminal CCN-51 and CCN-51 grafted on SCI-876. Furthermore, in inoculated plants the highest gs was found in the combination CCN-51 grafted on SIC-876. In contrast, in non-inoculated plants seminal CCN-51 showed the lowest *gs* value (Fig. 7).

It was also found that CCN-51 was the only genotype that did not show significant difference ($p < 0.05$) in leaf transpiration rate (*E*) in both inoculated and non-inoculated plants. For the other treatments, the *E* values were significantly

higher ($p < 0.05$) in plants without *M. perniciosa* inoculation compared to inoculated plants (Fig. 8).

In general, no significant difference ($p < 0.05$) between grafted and ungrafted treatments with and without inoculation were found for the instantaneous water use efficiency (*A/E*), except for CCN-51 grafted onto SIC-876 and infected with *M. perniciosa*. Infected seminal CCN-51 showed the lowest *A*/*E* values (Fig. 9).

Variables of chlorophyll fluorescence emission did not show, in general, significant differences ($p < 0.05$) among treatments. There was no significant difference in initial fluorescence (F_0), with the exception of significantly ($p < 0.05$) lower values for seminal CCN-51 plants inoculated with *M. perniciosa* and seminal not inoculated SCA-6 plants (Fig. 10A). Values of variable fluorescence (F_v) were significantly lower in inoculated seminal plants of the three genotypes studied and statistically similar in the grafted plants (Fig. 10B).

Regarding maximal fluorescence (F_m), significant variations among genotypes for plants inoculated with *M. perniciosa* were found. CCN-51 grafted on SIC-876 showed the lowest value (Fig. 11A). Among plants without inoculation, significantly highest values ($p < 0.05$) were determined in seminal CCN-51 and CCN-51 grafted on SIC-876, while the highest values were observed in CCN-51 grafted on SCA-6. In general, F_m values were significantly higher in plants without inoculation with *M. perniciosa* compared with values of non-inoculated plants, except for CCN-51 grafted onto SCA-6 (Fig. 11A). With regard to the maximum quantum yield of PS2 (F_v/F_m), the observed values in inoculated plants were significantly ($p < 0.05$) lower in seminal treatments and higher in CCN-51 x SIC-876 compared to non-inoculated plants. The combination CCN-51 x SCA-6 showed similar F_v/F_m values (Fig. 11B).

Grafted and non-grafted plants, with or without *M. perniciosa* inoculation, exhibited the typical transient OJIP kinetic curve for chlorophyll fluorescence emission at 60 days after inoculation (data not shown). However, with respect to electron flow parameters for the reaction center (Fig. 12), it should be noted that the absorption flow (*ABS/RC*) and electron capture (*TR/CR*) were not influenced by the presence of WB, except for seminal SCA-6 and CCN-51.

Mineral macro and micronutrients

In general, significant reductions ($p < 0.05$) were found in mineral macronutrient leaf concentrations of grafted or ungrafted inoculated plants, compared with plants without *M. perniciosa* inoculation (Table 1). Furthermore, grafted and ungrafted inoculated plants showed significant ($p < 0.05$) decrease in N concentration in relation to plants without WB. The exception was seminal SIC-876, whose N concentrations were similar in the presence or absence of WB and inoculated plants of CCN-51 grafted onto SCA-6, whose N concentrations were higher. In contrast, leaf P concentration showed a significant ($p < 0.05$) increase in plants with WB for seminal SIC-876 and SCA-6 and CCN-51 grafted on SIC-876 (CCN-51/SIC-876). No significant differences were observed in the other treatments for P concentration. In relation to K, the most significant decreases ($p < 0.05$) in concentration were observed in SIC-876 and CCN-51/SIC-876 with WB, compared with other treatments without WB (Table 1). Significant differences ($p < 0.05$) between grafted and ungrafted plants, with or without WB were found with regard to Ca and Mg concentration. Unlike Mg concentration, Ca concentrations were significantly higher in the grafted plants.

In relation to the mineral micronutrient leaf concentrations (Table 2), in general, no significant differences were observed between plants with or without WB regarding Fe and Zn concentrations, except for Fe in seminal SIC-876 and SCA-6, whose values were significantly ($p < 0.05$) higher in plants without WB. On the other hand, Fe concentration in inoculated CCN-51 grafted onto SIC-876 was significantly higher. Zn concentrations in seminal plants of SCA-6 and CCN-51 grafted on SIC-876 inoculated with *M. perniciosa* were also significantly ($p < 0.05$) higher. Leaf Cu concentrations were significantly higher in plants without WB of seminal SIC-876 and CCN-51 and CCN-51 grafted on SIC-876. However, showed higher values in SCA-6 inoculated with WB. Mn concentrations were significantly higher in non-inoculated seminal SIC-876 and inoculated CCN-51. CCN-51 grafted on both SIC-876 and SCA-6 showed similar Mn concentrations on both inoculated and non-inoculated plants (Table 2).

Discussion

Disease evaluation

CCN-51 showed differentiated responses regarding the rootstock in which it was grafted. The ID was significantly higher in CCN-51 grafted on SCA-6 than on SIC-876. Although SIC-876 had the highest ID, expected result since this genotype is reference for susceptibility, when used as rootstock delivered larger WB tolerance to the graft (CCN-51). This information, as far as we know, is unpublished and important for farmers that prepare grafted seedlings for planting of new areas or for replacing cacao crowns of susceptible plants through grafting of material of unknown origin, but tolerant to disease and high productivity, on susceptible rootstocks. Original cacao

plantations in Bahia, Brazil, were of Comum pedigree, which are susceptible to WB. On the other hand, SCA-6 maintained its known resistance pattern, confirming the possibility of their use as a source of resistance to WB [35].

Antioxidant metabolism

In the present study, SOD activity at the leaf level was significantly higher in seminal SCA-6, genotype used as a source of WB resistance. Furthermore, it was intermediate in seminal CCN-51, a tolerant genotype, and lower in seminal SIC-786 (Fig. 2). Interestingly, SOD activity on both grafted treatments was significantly lower. Therefore, seems that as a rootstock SCA-6 did not proportion protection through this enzyme to the scion. Actually, the inoculated CCN-51 grafted onto SIC-6 showed the lower activity value. SOD is one of the most effective intracellular antioxidants, thus, has been proposed as a key enzyme for stress tolerance in plants as it provides the first defense line against toxic effects of high ROS levels [36]. The enzyme removes $O_2^{\bullet-}$ catalyzing its dismutation, where an $O_2^{\bullet-}$ is reduced to H_2O_2 and the other oxidized to O_2 [37].

The lower CAT activity was found in seminal SIC-876, the genotype most susceptible to WB. However, it was higher in the CCN-51/SIC-876 combination; probably due to a scion effect. In the combination CCN-51/SCA-6 the higher activity values of the enzyme seems to be given by the resistant rootstock. CAT is another enzyme of the antioxidant system responsible for the conversion of H_2O_2 into O_2 and H_2O, also essential for removal of ROS [37]. CAT has one of the highest rates of detoxification of H_2O_2 compared to APX and GPX, being able to convert approximately 100 thousand molecules of H_2O_2 in H_2O and O_2 per s, without using a reducing substrate [20]. Typically, CAT reactions become more important when there

is a large H_2O_2 accumulation in cells; since, under normal concentrations H_2O_2 is reduced by glutathione reductase and peroxidases [20].

In our study, plants inoculated with *M. perniciosa* (with WB) used mainly peroxidases to remove the excess H_2O_2 produced, although, APX seem to have the leading role. This observation is drawn from the most APX activity in grafted and ungrafted genotypes infected with WB (Fig. 4). In the CCN-51/SIC-876 combination, seems that both scion and rootstock contribute for the higher activity values in the inoculated treatment. On the other hand, the importance of GPX is also seen in the CCN-51/SIC-876 combination (Fig. 5). The higher activity value of this enzyme in this combination compare to the levels shown by the resistant genotype (SCA-6) and appears that a synergistic effect is given by the interaction susceptible rootstock x tolerant scion. APX is considered the most important peroxidase in the elimination of excess H_2O_2 [20]. Furthermore, this enzyme has a higher affinity for H_2O_2 than CAT and GPX, and may have a more important role in the detoxification of ROS during stress [20,39]. Unlike CAT, APX and GPX transform H_2O_2 into H_2O and O_2 using substrates as reductant. APX uses ascorbate as electron acceptor and possess five known isoforms, among which some are present in thylakoids and glyoxysome membranes, and some in soluble forms in the chloroplast stroma [40,41]. On the other hand, GPX uses guaiacol as electron acceptor and possess important role in lignin biosynthesis [42,43].

Photosynthetic responses

Knowledge of limiting factors of photosynthesis (stomatal and not stomatal) is relevant to understand genotypic variations in response to biotic and abiotic stresses [44]. The lower values of *A* for grafted and ungrafted plants infected with WB

compared to the same treatment without inoculation demonstrated the stress caused by the infection of *M. perniciosa*, except for CCN-51 grafted on SIC-876. In this combination, both treatments showed the same *A* (Fig. 6). Therefore, seems that the combination of these two genotypes acted synergistically to cope with the attack of WB.

With the exceptions of both inoculated and non-inoculated CCN-51 and CCN-51 grafted on SIC-876, lower *gs* values were observed in the different treatments with WB (Fig. 7). Also, relatively high *E* values, although in general lower than non-inoculated plants, were observed in plants infected with WB. This indicates that the disease caused a decrease in the CO_2 fixation and transpiration rates, since changes in *gs* relate to the control of CO_2 assimilation and water loss, respectively [45]. Decreases in *gs* can restrict CO_2 fixation rates, with the consequent decrease in the concentration of CO_2 in substomatic and intercellular spaces [46]. The decrease in *E* reduces water loss, in the form of vapor, via stomata. The higher *A*/*E* value in the combination CCN-51 grafted onto SIC-876 and infected with *M. perniciosa*, in comparison to non-infected, was mainly due to the reduction in *E* values, since *A* and *gs* remained relatively constant. According to Kozlowski and Pallardy [47], stomatal closure entails proportionally greater decreases in *E* than in *A*, in face of additional resistances associated with the diffusion of CO_2 in relation to resistances of water in the leaf.

Through kinetics analysis of transient fluorescence of chlorophyll (OJIP curve) can be obtained information on the structural and functional parameters that quantify the performance of the photosynthetic machinery during the photochemical phase [49,50]. In the present study, the decrease in fluorescence emission kinetics in some treatments infected with WB, and its increase in the same treatments without

infection, show the existence of damage at the PS2 level in the inoculated plants. This observation is supported by lower values of *ABS*/*RC* and *TR*/*RC*, related to absorption flux and electron capture, respectively, at the PS2 level mainly in SCA-6 (Fig. 12).

In this study, F_m showed higher values in the treatments in which plants were infected without WB compared to infected plants, except for the combination CCN-51/SCA-6, which values on both infected and non-infected plants were similar. The maximum fluorescence emission intensity is shown when virtually all Q_A is reduced and reaction centers are unable to increase photochemical reactions, reaching its maximum capacity [51]. Differences in the F_m values may show variations in the properties of electron acceptors of PS2, caused by conformational changes induced by stress in the main constituent of the protein complex, the D_1 protein, which forms the PS2 [52]. The increase in F_m values can be attributed to protein phosphorylation in the thylakoid membranes [53].

In this work, we observed for the seminal genotypes infected with WB that the F_v values were significantly lower when compared with the treatments in which the plants were not infected with *M. perniciosa*. On the other hand, both grafted treatments (inoculated and non-inoculated) showed statistically similar F_v. The same effect was also observed for F_0 values in seminal CCN-51 and SCA-6 infected with WB. The decrease in F_v may indicate damages to PS2 reaction center associated with the reduction of photosynthetic electron transport to PS2 [54]. F_0 values indicate the fluorescence emitted when Q_A is fully oxidized and the PS2 reaction center is open [51]. On the other hand, a decrease of F_0 indicates that there was an increase in electron flow through PS2 [55]. F_0 values may increase if the PS2 reaction center

were compromised or if the transfer of excitation energy from the antenna-complex to the reaction center is damaged [51].

The ratio F_v/F_m is considered an important indicator of the effects of environmental stresses on photosynthesis [48]. F_v/F_m values are almost constant for most plant species under non-limiting conditions, with values around 0.832 ± 0.004 [56]. In this study, F_v/F_m values were lower in the seminal genotypes infected with WB, compared to uninfected plants suggesting and inefficient conversion of light energy at the PS2 level for ATP and NADPH production [57,22]. Although with low F_v/F_m values, in both grafted treatments the interaction scion:rootstock acted differently showing lower effect of the infection on both combinations. Direct effects of the infections are seen in the inoculated seminal genotypes. In these treatments low F_v/F_m values indicate the occurrence of photoinhibition or damage to the PS2 antenna complex [58].

Mineral macro and micronutrients

In our study significant reductions were observed in leaf N concentration in plants infected with *M. perniciosa* (Table 1). Nitrogen deficiency can cause leaf chlorosis, delay of vegetative growth, early leaf senescence and problems related to morphological characteristics, leading to reduction in the shoot/root ratio. This is probably due to reduced transpiration and lower N transport from roots to shoots [59]. This was observed in this study. Transpiration rate reductions were found in treatments infected by *M. perniciosa*. Furthermore, the yellowing of mature brooms, followed by necrosis was evident.

Unlike N, an increase of P in seminal SIC-876 and SCA-6 and in CCN-51 grafted on SIC-876 and infected with *M. perniciosa* was observed (Table 1). This

element is responsible for the storage and transfer of energy and comprises esters of carbohydrates, nucleotides and nucleic acids. Under stress conditions, plants may show alterations in the concentrations of P, with positive effects on increasing water use efficiency and stomatal conductance [60]. This, in turn, may have contributed to the reduction of *E* and the increase of *A*/*E* in CCN-51 grafted on SIC-876 and infected with *M. perniciosa*.

It was also observed a reduction in leaf K concentration in seminal SIC-876 and CCN-51 grafted on SIC-876, both infected with *M. perniciosa*. This is an essential macronutrient for plant growth and development. In *T. cacao* K represents near 70% of the nutrients in the xylem sap [61]. It plays a crucial role in various metabolic processes by regulating the osmotic potential. It is required for enzyme activity and protein and carbohydrate syntheses, assists in the opening and closure of stomata and participates in water relations and cell elongation. Therefore, K deficiency slows plant growth, promotes leaf chlorosis, necrotic spots and shortening of internodes [62]. Genotypic differences in the capacity of K utilization can be attributed to differences in (i) partitioning and redistribution of K at the cellular or whole plant levels, (Ii) substitution of K by other ions, (Iii) or partitioning of resources for a more economical production [63,64].

Regardless of presence or absence of WB, significant differences were observed among grafted and ungrafted plants regarding Ca and Mg concentrations at the leaf level (Table 1). Unlike Mg concentration, Ca content was significantly higher in plants grafted with CCN-51, unequivocally showing the interaction scion: rootstock. Even though Ca plays a structural role in cells and signals some plants responses to the environment [65], this increase appears not to be involved in protection to the cacao plants.

Magnesium is an essential component for the activation of many enzymes, including ATPases, protein kinases and phosphatases [66]. The stability of ribosomes and photosynthesis can be affected by Mg^{2+} deficiency since this element is the central atom of the chlorophyll molecule and participates in the aggregation of ribosomes, besides acting on the stability of cell membranes [67,68]. In our study, regardless of being inoculated or not, the grafted plants showed significantly lower values of leaf Mg concentration in comparison of the ungrafted treatments, which also shows the interaction scion: rootstock.

In general in this study, no significant differences were observed between plants with or without WB regarding Fe and Zn concentrations at the leaf level. However, inoculated plants of CCN-51 grafted on SIC-876 showed significantly higher concentrations of Fe compared to non-inoculated plants of that combination. Iron has an important role as a component of enzymes involved in electron transfer (redox reactions) like cytochromes. Besides, it is needed for the synthesis of some chloroplast complexes made from chlorophyll and proteins [45]. Interestingly, Zn concentration in plants of SCA-6 and CCN-51 grafted on SIC-876 and infected with WB were significantly higher than in the other treatments. Zinc is required for the activity of various enzymes and chlorophyll biosynthesis in some plant species [69]. The isoenzyme Cu-Zn superoxide dismutase (Cu-Zn-SOD), which contains Zn as a cofactor, plays an important role in the removal of superoxide radical ($O_2^{\bullet}$) and thus protects the membranes and proteins against oxidation [70].

Copper concentration was significantly higher in seminal plants of SCA-6 and CCN-51 and CCN-51 grafted on SIC-876 without WB. This is in contrast to Zn behavior; Cu concentration was lower and Zn higher in inoculated plants. Copper is absorbed by plants in very small quantities, because its requirement is relatively low

[71]. It is involved in the biosynthesis of polyphenol oxidase and is also a cofactor of Cu-Zn-SOD. Its excess inhibits the activity of a number of enzymes and interferes in various aspects related to plant biochemistry, including photosynthesis, pigment synthesis, metabolism of fatty acids and proteins, respiration, N fixation and membrane integrity [72].

Manganese concentrations were significantly higher in seminal CCN-51 and CCN-51 grafted on both SIC-876 and SCA-6, although with no differences between inoculated and non-inoculated plants. Apparently the higher concentration of Mn in the grafted treatments comes from the ability of the scion (CCN-51) to uptake this element, showing the effects of the interaction. However, seems that did not provide protection to the inoculated grafted treatments, since, Mn has catalytic role in superoxide dismutase activity for the protection of plants against ROS [73]. Its deficiency, evidenced by interveinal chlorosis in young leaves, can harm plant growth and affects the photosynthetic oxygen evolution system.

Conclusions

The interactions between scion and rootstock in the combination CCN-51/SIC-876 made the graft most tolerant to *M. perniciosa* infection. SIC-876 rootstock, considered susceptible to witches' broom, had a positive effect on the performance of CCN-51 graft with respect to tolerance to the disease.

SIC-876 rootstock provided greater tolerance to the CCN-51 graft to witches' broom, mainly due to increased APX and GPX activities.

The selective uptake of Ca and Mg are proof of the existence of interaction between scion and rootstock in the *T. cacao* combinations tested.

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Figure Legends

Fig. 1. Mean values of symptoms (A) and disease index (DI) (B) in grafted and ungrafted *T. cacao* genotypes at 60 days after inoculation with *M. perniciosa*. Columns represent means and bars the standard error (n = 4). Letters indicate comparison between treatments by Tukey's test ($p < 0.05$).

Fig. 2. Superoxide dismutase (SOD) activity in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material occurred 48 h after plant inoculation. Columns represent means and bars standard errors (n = 4). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants inoculated or not inoculated by t test ($p < 0.05$).

Fig. 3. Catalase (CAT) activity in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material occurred 48 h after plant inoculation. Columns represent means and bars standard errors (n = 4). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants inoculated or not inoculated by t test ($p < 0.05$).

Fig. 4. Ascorbate peroxidase (APX) activity in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material occurred 48 h after plant inoculation. Columns represent means and bars standard errors (n = 4). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants inoculated or not inoculated by t test ($p < 0.05$).

Fig. 5. Guaiacol peroxidase (GPX) activity in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material occurred 48 h after plant inoculation. Columns represent means and bars standard errors (n = 4). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants inoculated or not inoculated by t test ($p < 0.05$).

Fig. 6. Net photosynthetic rate (A) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean

comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 7. Stomatal conductance to water vapor (gs) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 8. Transpiration rate (E) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 9. Instantaneous water use efficiency (A/E) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 10. Initial fluorescence (F_0) (A) and variable fluorescence (F_v) (B) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation on the same leaves used for gas exchange determinations. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 11. Maximal fluorescence (F_m) (A) and maximum quantum efficiency of photosystem 2 (F_v/F_m) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation on the same leaves used for gas exchange determinations. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test

($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 12. Specific absorption flows (*ABS*/*RC*) (A) and electron capture (*TR*/*RC*) (B) in leaves of grafted and not grafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Measurements were made at 60 days after plant inoculation on the same leaves used for gas exchange determinations. Columns represent means and bars standard errors (n = 8). Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).

Fig. 1

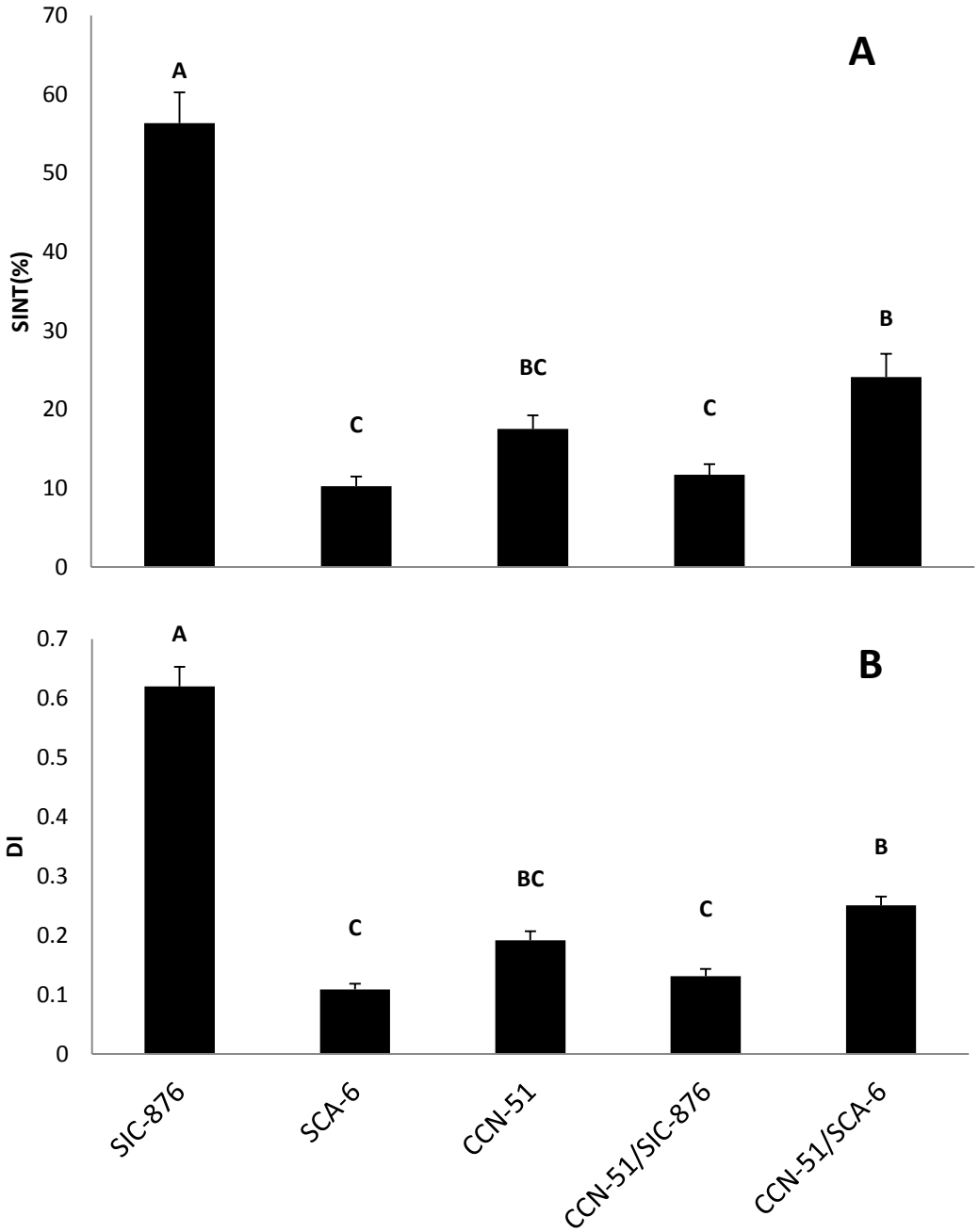
A
SINT(%)
70
60
50
40
30
20
10
0
A
C
BC
C
B
B
DI
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
C
BC
C
B
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 2

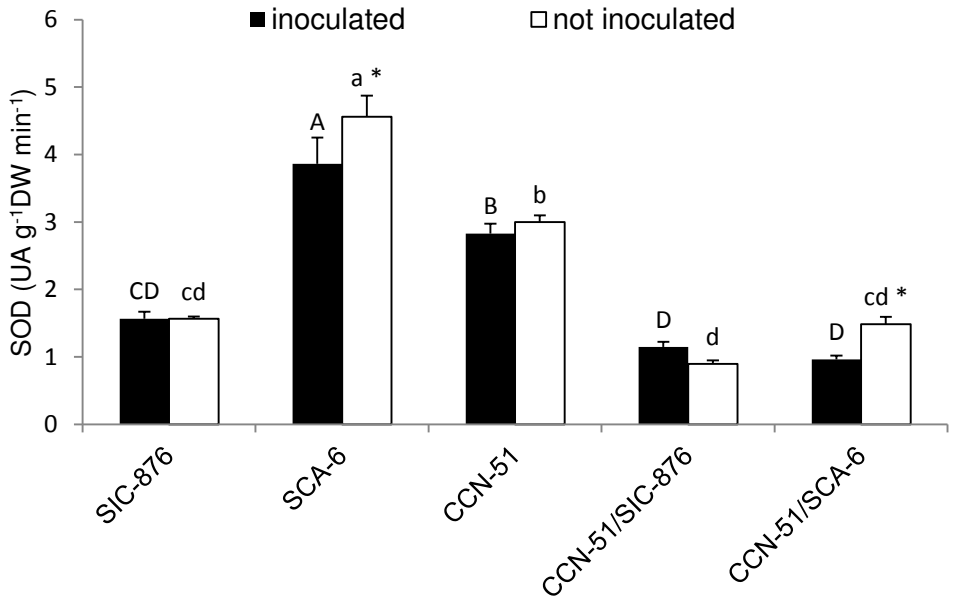
inoculated
not inoculated
SOD (UA g-1DW min-1)
0
1
2
3
4
5
6
CD
cd
A
a *
B
b
D
d
D
cd *
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 3

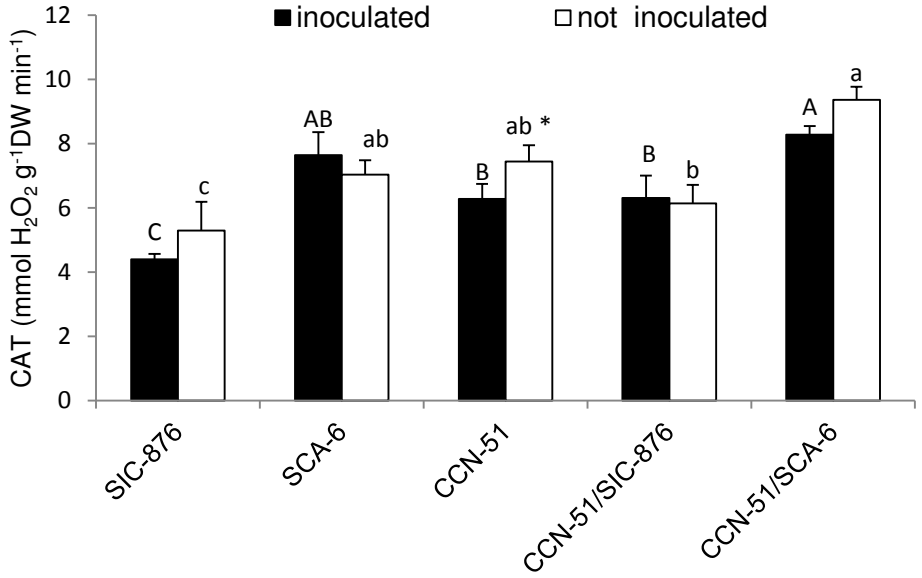
inoculated
not inoculated
CAT (mmol H2O2 g-1DW min-1)
0
2
4
6
8
10
12
C
c
AB
ab
B
ab *
B
b
A
a
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 4

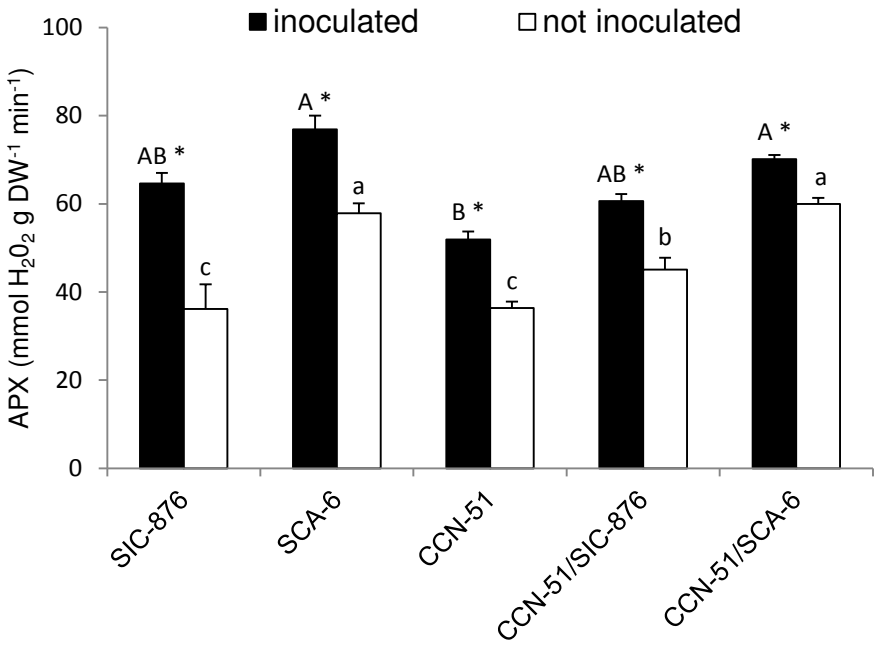
inoculated
not inoculated
APX (mmol H_2O_2 g DW^{-1} min^{-1})
100
80
60
40
20
0
AB *
c
A *
a
B *
c
AB *
b
A *
a
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 5

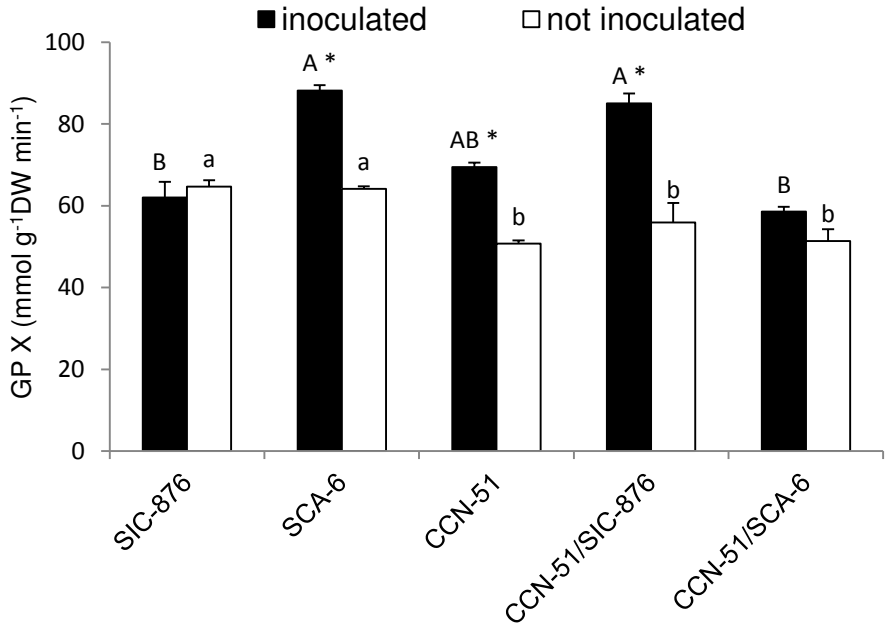
inoculated
not inoculated
GP X (mmol g^{-1}DW min^{-1})
100
80
60
40
20
0
B
a
A *
a
AB *
b
A *
b
B
b
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 6

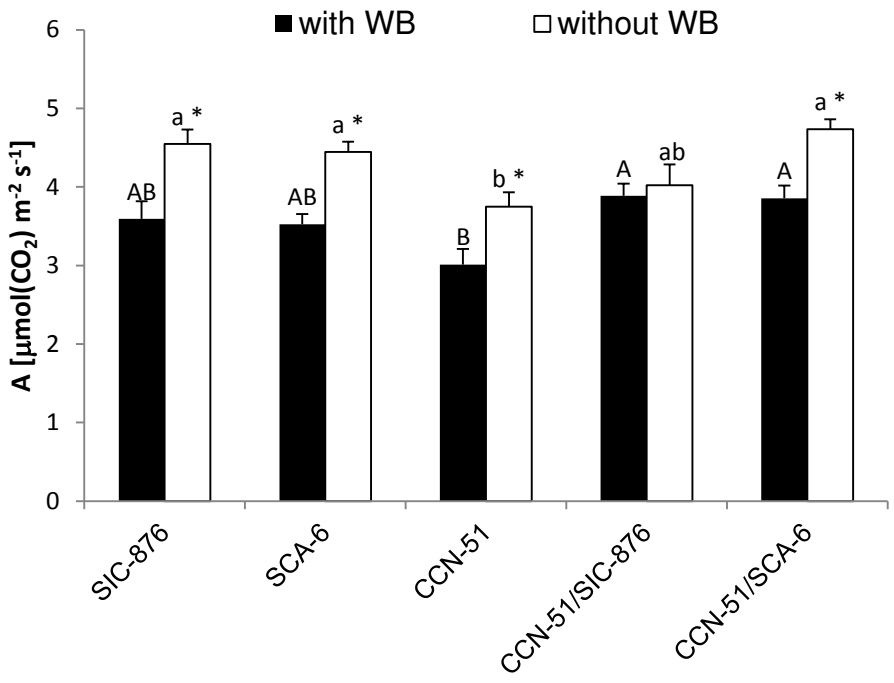
with WB
without WB
A [µmol(CO2) m-2 s-1]
0
1
2
3
4
5
6
AB
a *
AB
a *
B
b *
A
ab
A
a *
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 7

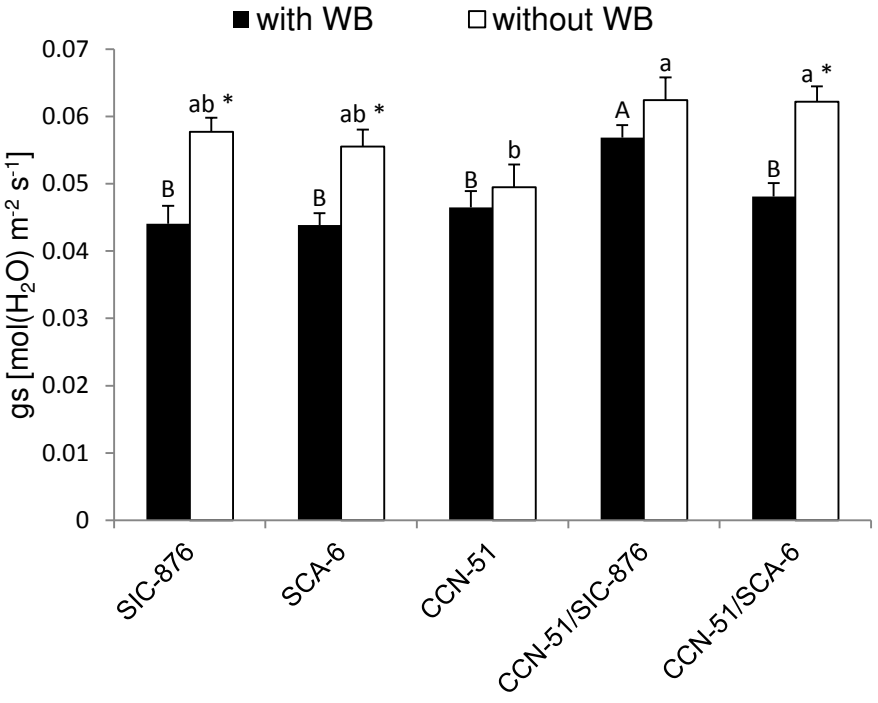
with WB
without WB
gs [mol(H2O) m-2 s-1]
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
B
ab *
B
ab *
B
b
A
a
B
a *
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 8

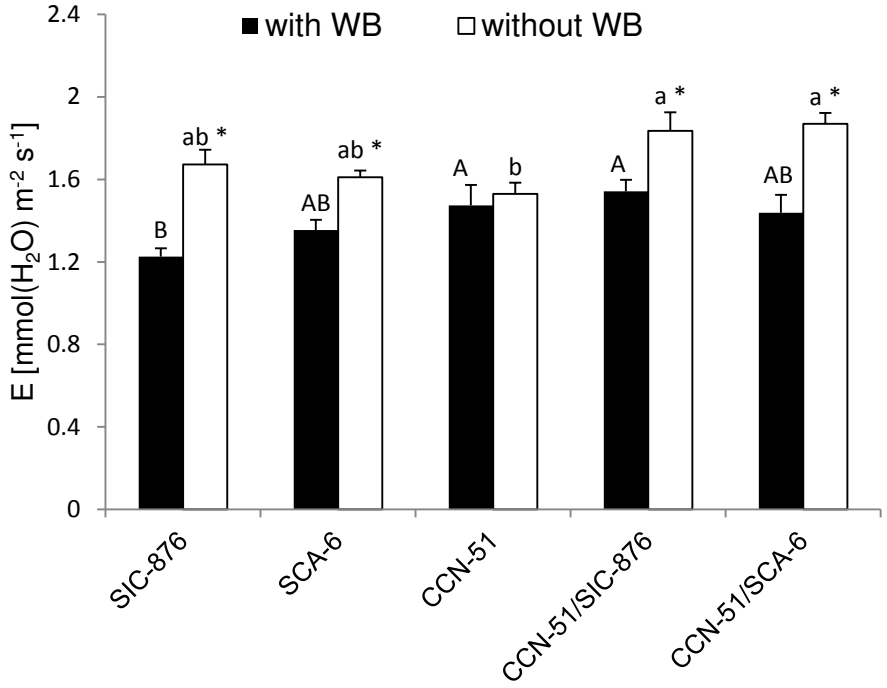
with WB
without WB
E [mmol(H2O) m-2 s-1]
2.4
2
1.6
1.2
0.8
0.4
0
B
ab *
AB
ab *
A
b
A
a *
AB
a *
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 9

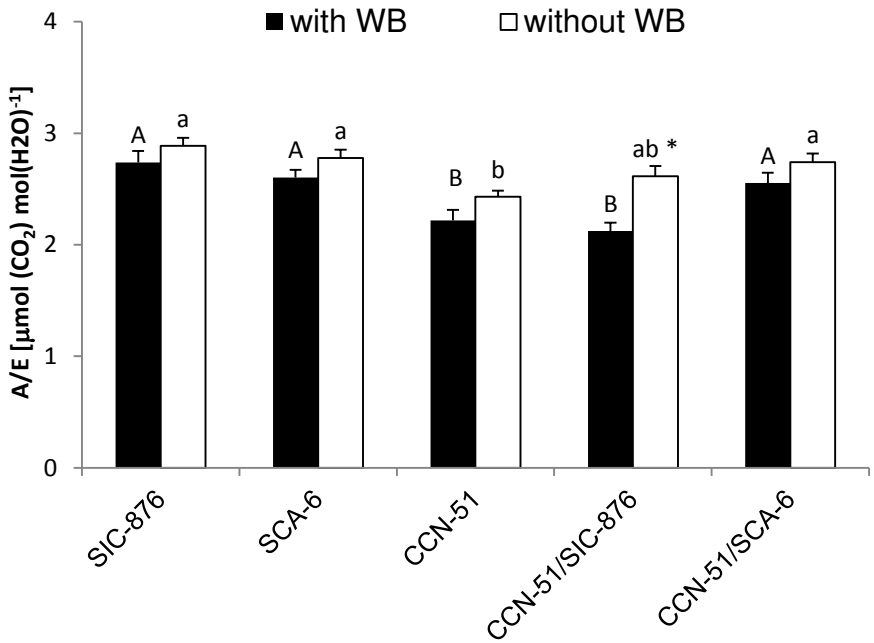
with WB
without WB
A/E [µmol (CO2) mol(H2O)-1]
4
3
2
1
0
A
a
A
a
B
b
B
ab *
A
a
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 10

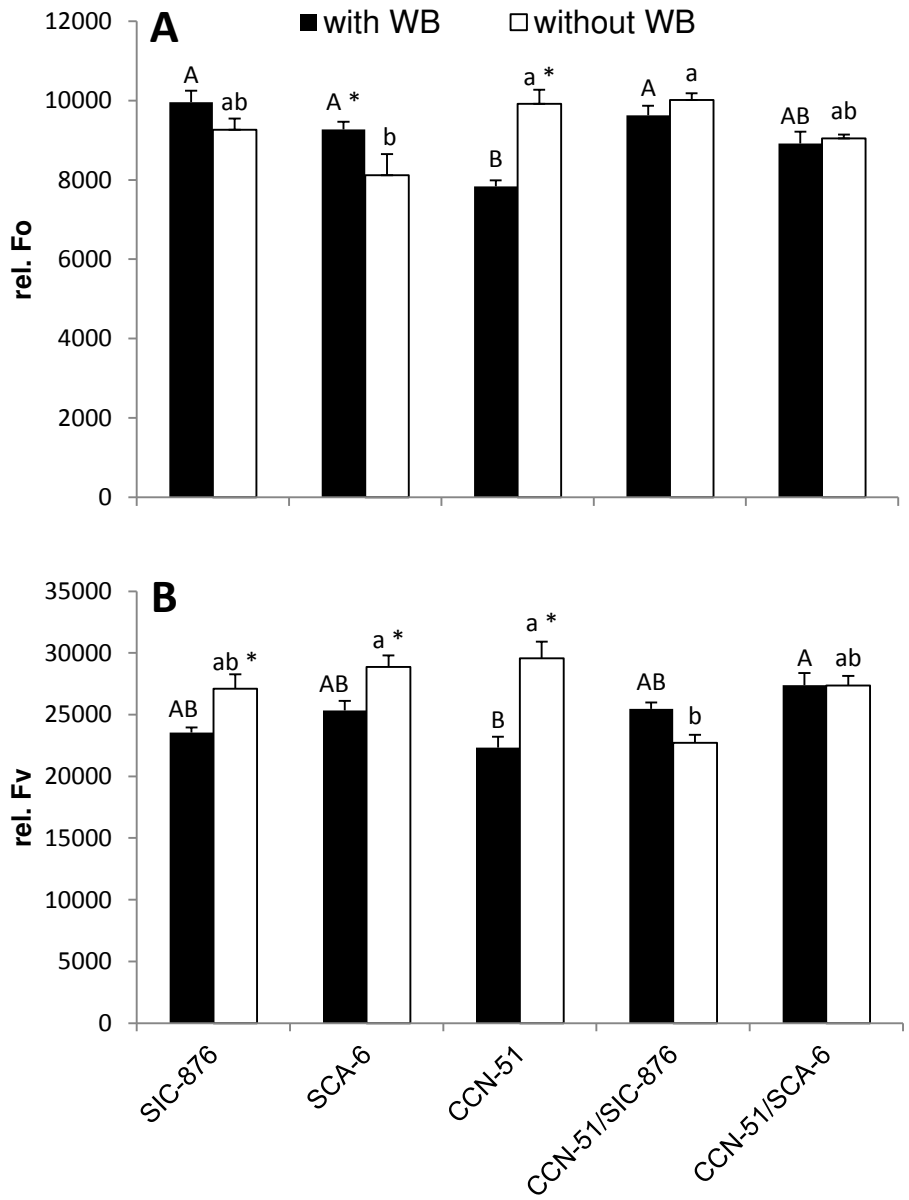
A
with WB
without WB
rel. Fo
12000
10000
8000
6000
4000
2000
0
A
ab
A *
b
B
a *
A
a
AB
ab
B
35000
30000
25000
20000
15000
10000
5000
0
rel. Fv
AB
ab *
AB
a *
B
a *
AB
b
A
ab
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 11

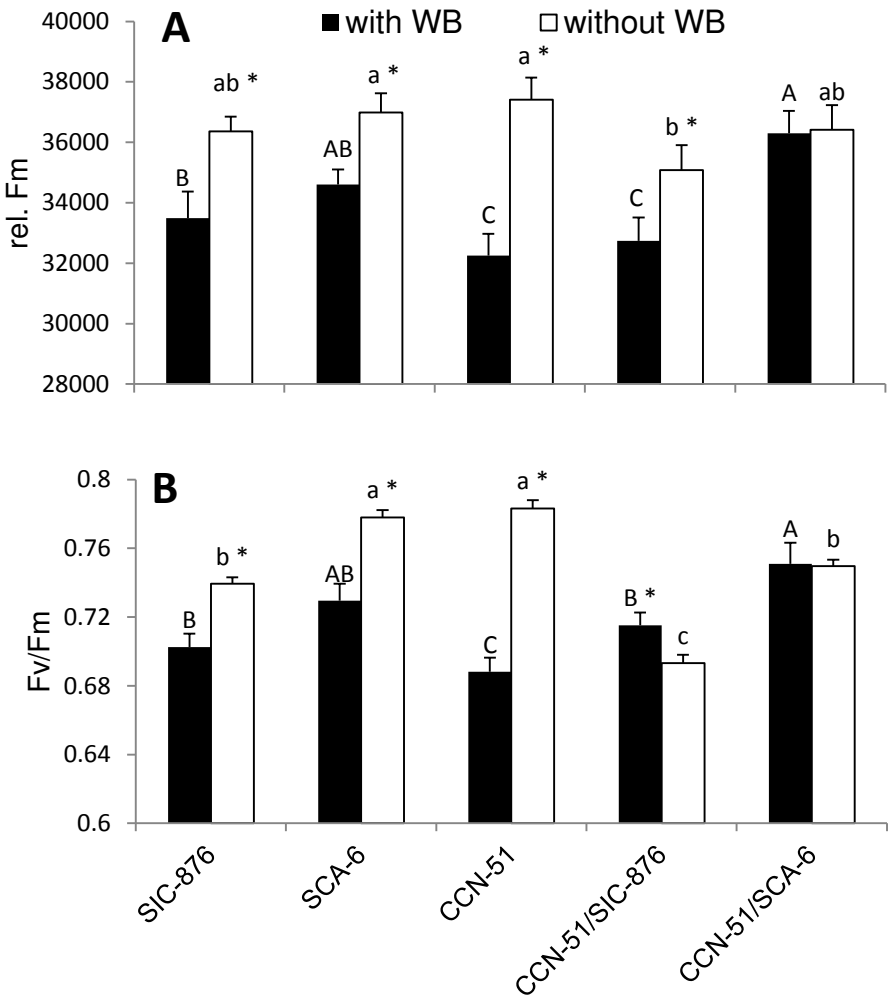
A
with WB
without WB
rel. Fm
40000
38000
36000
34000
32000
30000
28000
B
AB
C
C
A
ab *
a *
a *
b *
ab
B
Fv/Fm
0.8
0.76
0.72
0.68
0.64
0.6
b *
a *
a *
B *
A
b
c
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Fig. 12

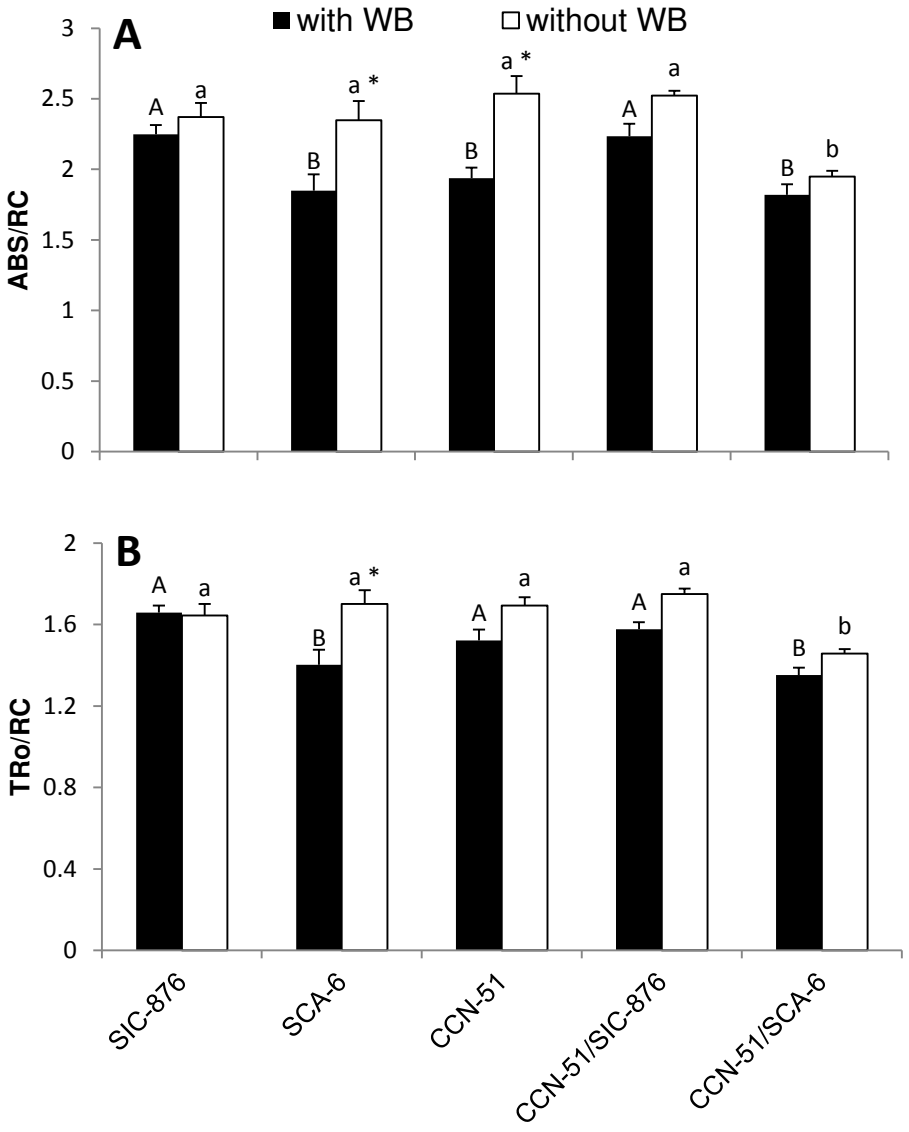
A
with WB
without WB
ABS/RC
3
2.5
2
1.5
1
0.5
0
A
a
B
a *
B
a *
A
a
B
b
B
TRo/RC
2
1.6
1.2
0.8
0.4
0
SIC-876
SCA-6
CCN-51
CCN-51/SIC-876
CCN-51/SCA-6

Table 1. Mineral macronutrient concentrations in leaves of grafted and ungrafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material was done at 60 days after plant inoculations.

		mg plant^{-1}			
Genotype	WB	N	P	K	Ca	Mg
SIC-876	with	964 ± 15.5 A	143 ± 4.2 A *	728 ± 26 A	251 ± 2.4 D	309 ± 3.1 B
	without	999 ± 2.2 a	114 ± 3.5 c	853 ± 39 a *	272 ± 1.2 b	361 ± 8.3 a *
SCA-6	with	833 ± 12.2 B	151 ± 4.6 A *	705 ± 48 A	263 ± 4.4 D	423 ± 2.0 A *
	without	957 ± 19.5 ab *	129 ± 3.1 b	737 ± 30 b	278 ± 5.1 b	355 ± 7.4 a
CCN-51	with	767 ± 18.9 C	120 ± 7.5 C	750 ± 33 A *	687 ± 36.1 C *	226 ± 14.2 C
	without	1047 ± 19.4 a *	122 ± 3.3 b	683 ± 20 b	281 ± 11.9 b	356 ± 7.3 a *
CCN-51/SIC-876	with	835 ± 15.1 B	143 ± 7.8 A *	646 ± 40 B	834 ± 40.2 B	205 ± 0.8 C
	without	961 ± 20.2 ab *	127 ± 5.1 b	792 ± 35 ab *	1011 ± 60.5 a *	204 ± 1.0 b
CCN-51/SCA-6	with	887 ± 27.7 AB *	130 ± 4.5 B	550 ± 28 C	1114 ± 40.9 A	204 ± 1.3 C
	without	810 ± 17.8 b	140 ± 5.1 a	570 ± 19 c	1113 ± 25.1 a	204 ± 1.1 b

[a] Capital letters indicate comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).
(Means ± SE; n = 4).

Table 2. Mineral micronutrient concentrations in leaves of grafted and ungrafted and inoculated and not inoculated with *M. perniciosa T. cacao* genotypes. Sampling of plant material was done at 60 days after plant inoculations.

		mg plant^{-1}		
Genotype	WB	Fe	Zn	Cu	Mn
SIC-876	with	9.3 ± 1.2 A	20 ± 0.3 B	1.1 ± 0.05 C	64 ± 3.3 C
	without	11 ± 2.2 a *	21 ± 1.0 a	1.2 ± 0.05 c *	179 ± 1.4 a *
SCA-6	with	6.6 ± 0.3 B	20 ± 0.6 B *	1.4 ± 0.03 A *	78 ± 2.1 C
	without	7.6 ± 0.4 b *	16 ± 0.5 c	1.2 ± 0.08 c	68 ± 3.4 c
CCN-51	with	7.3 ± 0.3 B	18 ± 1.3 C	1.2 ± 0.05 B	141 ± 9.3 A *
	without	7.2 ± 0.8 b	19 ± 0.4 b	1.6 ± 0.03 a *	96 ± 1.9 b
CCN-51/SIC-876	with	6.6 ± 0.1 B *	23 ± 0.2 A *	1.1 ± 0.06 C	127 ± 5.4 AB
	without	5.4 ± 0.1 c	17 ± 0.6 b	1.4 ± 0.03 bc *	118 ± 6.0 b
CCN-51/SCA-6	with	5.1 ± 0.3 C	17 ± 0.6 C	1.1 ± 0.07 C	107 ± 5.1 B
	without	5.1 ± 0.2 c	17 ± 0.4 b	1.1 ± 0.03 c	111 ± 5.1 b

[a] Capital letters comparison between grafted and ungrafted genotypes by Tukey's test ($p < 0.05$); lower case letters indicate mean comparisons of plants with or without symptoms of witches' broom by t test ($p < 0.05$).
(Means ± SE; n = 4).

4. CAPÍTULO II

Photosynthetic and Nutritional Responses of *Theobroma cacao* Genotypes Submitted to Induction of Resistance to *Moniliophthora perniciosa* by Chemicals Elicitors

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*alexalan@uesc.br

Abstract

Cacao (*Theobroma cacao* L.) is one of the most important perennial crops in the world. Witches' broom (WB) is the main cacao disease in Brazil and one of the causes of the cacao crisis in South Bahia due to cost increase and crop yield reductio. Witches' broom is caused by the basidiomycete *Moniliophthora perniciosa*. With the objective to evaluate the effect of resistance inducer for WB in cacao, an experiment was conducted under greenhouse condition using two seminal genotypes

of *T. cacao*, a susceptible (SCI-876) and a tolerant (CCN-51) to WB. The clone CCN-51 is currently recommended to farmers also for its high productivity and higher seed fat content. The seedlings were treated with distilled water (control), sucrose, glucose and salicylic acid (SA). At 60 days after planting the seedlings were inoculated with viable basidiospores (2 x 10^5 spores mL^{-1}) of *M. perniciosa*. The responses were evaluated at the leaf level through determination of mineral macro and micronutrient concentrations, measurements of gas exchange and chlorophyll fluorescence. In both genotypes, seedlings treated with SA had significantly lower disease indices. In SIC-876, sucrose showed similar results. Leaf concentrations of Fe and Mn were significantly lower in both genotypes in plants treated with SA, in contrast, in this treatment were observed the highest instant water use efficiencies. There were significant differences among genotypes with respect to fluorescence parameters; CCN-51 showed the highest quantum yield values of photosystem 2. The use of the chemical elicitors used minimized the effects of WB in both genotypes. SA was the most efficient elicitor in induction of resistance in both genotypes studied.

Keywords: witches' broom, systemic acquired resistance, gas exchange

4.1 Introduction

Cacao (*Theobroma cacao* L.) is a typical woody species of tropical climate, diploid (2n = 20), perennial and preferentially allogamous. Among the 22 species that make up the genus, just cacao and cupuassu (*T. grandiflorum* L.) are exploited commercially in Brazil (Monteiro; Ahnert, 2012; Almeida; Valle, 2007). *T. cacao* is explored mainly for chocolate manufacture; however, it can also be used for production of cosmetics, beverages, jellies, creams and juices (Almeida; Valle, 2007). Worldwide cacao production involves about two million farmers in more than 50 countries (Knight, 2000). Brazil, one of the world's leading producers, showed a crop forecast for 2014 of 281,000 tons, with Bahia, the main producing state with an estimate of over 179,000 tons, accounting for more than 64% of the total cacao production in the country (IBGE, 2014).

In 1989, Witches' Broom (WB) a disease caused by the fungus *Moniliophthora perniciosa* (Aime; Phillips-Mora, 2005), was first recorded in the cacao region of Bahia (Pereira et al., 1989). Until the late 1990s, the Bahia cacao region was formed by a large mass of contiguous areas and varieties, most of them susceptible to WB; therefore, the disease spread rapidly throughout the region. Its spread caused a drastic drop in production, which decreased from 400,000 tons per year to 100,000 (Midlej; Santos, 2012). It also caused a drastic change in the socioeconomic and environmental scenario, given that the cacao activity has been for a long time, the main source of income in this region (Pereira et al., 1989). Since then, many scientific and also practical advances were achieved for cacao production; however there is still a lack of a lasting and permanent solution for its control. However,

resistant varieties were developed as well as management techniques to reduce production losses (Monteiro; Ahnert, 2012, Pereira; Valle, 2012).

Faced with this crisis situation of the Brazilian cacao crop, alternative methods and/or the use of integrated management for WB have been targeted (Bastos, 2004; Silva, Bastos, 2007; Pereira; Valle, 2012), among them resistance induction with biotic or abiotic products (Resende et al 2002; Vieira and Valle, 2012).

The induced resistance (IR) in plants is activated when inducers bind to receptor molecules located on the plasma membrane of the plant cell, triggering the activation of various defense mechanisms (Resende et al, 2002; Vieira and Valle, 2012). RI can be divided into two categories: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (van Loon et al., 1998). SAR is a natural defense mechanism of plants against diseases first observed by botanists in the early twentieth century (Chester, 1933). The plant uses pattern recognition receptors to distinguish microbial conserved sequences that trigger an immune response (Song et al., 1995). Induced resistance is a physiological state in which plant defenses are increased by a specific elicitor enhancing its defenses against subsequent biotic challenges (Devendra et al., 2007).

In general, the elicitors commonly isolated and identified belong to different chemical classes such as carbohydrates, peptides, proteins, lipids, glycoproteins, glycopeptides and fatty acids (Nürnberger; Brunner, 2002; Walters et al., 2005). Also, studies with different crops, including cacao, has demonstrated inducing effect of various nutrients and micronutrients in increased resistance to diseases since they act as cofactors for enzymes involved in the synthesis of phenolic compounds, such as manganese (Silva et al., 2008). Sugars such as glucose and sucrose are recognized as signaling molecules in plants (Bolouri-Moghaddam et al., 2010).

Cacao treated with potassium chloride, glucose, sucrose and salicylic acid demonstrated effective action in inducing resistance to WB (Vieira, Valle, 2006). This study, therefore, aimed to evaluate the effect of chemical elicitors (glucose, sucrose and salicylic acid) as inducers of resistance to WB in cacao, through the determination of symptoms and disease severity, concentration of leaf macro and micronutrients minerals and leaf gas exchange in a tolerant and a susceptible to WB genotypes.

4.2. Materials and methods

4.2.1. Plant material and growth conditions

We evaluated two cacao genotypes with different characteristics in regard to tolerance to WB: SIC-876 (susceptible) and CCN-51 (tolerant). The study was conducted in a greenhouse of the Plant Physiology Section (SEFIS), at the Cocoa Research Center (CEPEC), main research unit of the Executive Commission for the Cacao Farming Plan (CEPLAC) located in Ilhéus county, state of Bahia, Brazil (39º13'59 "O, 14º45'15" S, 55 m above sea level).

Seeds obtained from pods produced through open pollination at the active germplasm bank (BAG) of CEPEC were pre-germinated for 48 h in sterile moistened sawdust. After this period, were planted in plastic tubes containing the commercial substrate Plantmax™ and maintained under greenhouse conditions.

Inductors application

Thirty days after germination, the seedlings were induced by application of sucrose (154.0 g L^{-1}), glucose (108.1 g L^{-1}), salicylic acid (0.8 g L^{-1}) and 2.0 mL of distilled water per plant (control) on the abaxial leaf surface using a small sprayer.

Inoculum Production

Inocula of *M. perniciosa* CPK were produced at the Molecular Plant Pathology Laboratory of CEPEC/CEPLAC. For basidiomata production, infected branches (dry brooms) were collected and kept in a mist chamber maintained at 100% relative humidity. From the basidiomas produces basidiospores were collected and preserved in 16% glycerol solution (Frias et al., 1995) and stored in liquid nitrogen for later use.

Inoculation

After application of the inductors, the seedlings were grown under greenhouse conditions for 20 d, when, half were inoculated with basidiospores. However, the day before, the plantlets were taken to a chamber with temperature around 25°C and relative humidity near 100%, then 20 µL of a suspension of $2x10^5$ basidiospores mL^{-1} were applied on the apical bud. The seedlings used as controls received instead of the suspension a 0.2% water-agar drop. All plants were maintained for 24 h in the climatized chamber. After this period, were transferred to a greenhouse and irrigated daily with water and once a week with 50 mL of Arnon and Hoagland solution (1950).

4.2.2. Disease evaluation

We evaluated the symptoms presented by the seedlings individually at 30 and 60 days after inoculation (DAI) observing the type of broom, the amount of terminal and axillary brooms and the diameter and height of terminal brooms. Two variables were used: the percentage of plants showing any disease symptoms (SINT), such as terminal, axillary, cotyledonar and/or dry brooms, stem, hypocotyl, petiole and/or pulvinus swelling, canker, multiple shoots or hypertrophy (Figure 1). The other

variable was the disease index (DI) calculated by ID = VT + VA + (0.5 * CVT) + NVA, wherein VT = number of terminal brooms, VA = number of axillary brooms, CVT = length of terminal broomstick and NVA = number of axillary broom larger than 1 cm.

4.2.3 Concentration of mineral macro and micronutrients

Concentrations of mineral nutrients were evaluated in the leaf dry biomass collected 60 DAI. After nitropercloric digestion of samples determinations of Ca, Mg, Fe, Zn, Cu and Mn were done by atomic absorption spectrophotometry, P by colorimetry, using the Vitamin C method (Braga; Defelipo 1974) and K by flame emission photometry. The N concentration was determined by the Kjeldahl method, after sulphosalicylic sample digestion (Jackson, 1958).

4.2.4. Leaf gas exchange

At 30 and 60 DAI, measurements were made of gas exchange in mature, fully expanded leaves (2nd or 3rd leaf from the plagiotropic apex). The measurements were made between 08:00 and 12:00 h, in all treatments including the control plants with or without symptoms (brooms). The measurements were performed using a portable photosynthesis system model Li-6400 (Li-Cor Biosciences Inc., NE, USA) equipped with a source of artificial light 6400-02B RedBlue adjusted to 800 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR). The minimum time provided for stabilization of readings was 60 s and the maximum to save each reading was 120 s. The maximum permitted coefficient of variation to save each reading was 0.3%. In addition to PAR, were kept constant atmospheric CO_2 inside the chamber and the block temperature at 26°C. CO_2 flow rate was adjusted to maintain a concentration of 380 µmol CO_2 mol^{-1} air inside the chamber. The net

photosynthetic (*A*) and transpiration (*E*) rates by unit leaf area, stomatal conductance to water vapor (*gs*) and the ratio between the internal and atmospheric CO_2 concentrations (*Ci*/*Ca*) was estimated from the amounts of CO_2 and humidity variations within the chamber, as determined by the infrared gas analyzer of the apparatus at PAR ≥ 400 µmol photons m^{-2} s^{-1}. Instant (*A*/*E*) and intrinsic (*A*/*gs*) water use efficiencies were also calculated, as well as carboxylation efficiency (*A*/*Ci*).

4.2.5. Chlorophyll fluorescence

Fluorescence measurements of chlorophyll **a** were made in the same leaves used for the determinations of gas exchanges between 08:00 and 12:00 h, with a total of eight measurements per treatment. The determinations were made using a portable fluorometer (Handy-PEA, Hansatech Instruments Ltd., Norfolk, UK). The selected leaves were dark adapted for a period of at least 20 min, to reflect incident solar radiation, decrease leaf temperature and oxidize the photosynthetic electron transport system, using appropriate clips. After dark adaptation, the leaves were exposed to a saturating light pulse (3000 µmol photons m^{-2} s^{-1}, wavelength 650 nm for 1 s) and the fluorescence emission signals were recorded in the data acquisition system of the equipment using specific software.

The fluorescence intensity measured at 50 µs was considered as the initial fluorescence (F_0). From the fluorescence intensity of chlorophyll **a**, the parameters proposed by the JIP test were calculated (Strasser; Strasser, 1995; Michael-Tsimilli; Strasser, 2008), using the Biolyzer software (Laboratory of Bioenergetics, University of Geneva, Switzerland).

4.2.6. Statistical analysis

The seedlings were arranged in a completely randomized design, with two genotypes, eight treatments, four replicates and 30 plants per experimental unit. Analysis of variance and comparison of treatment means by Tukey test at $p < 0.05$ were perform. For leaf gas exchange and chlorophyll fluorescence data, mean comparisons between plants with and without WB were made using Students' t test ($p < 0.05$).

4.3. Results

4.3.1 Symptoms and disease index

Significant differences were found ($p < 0.05$) among genotypes and the evaluated chemical elicitors with respect to the proportion of brooms (SINT) and disease index (ID) (Figure 2). As expected, the values of both SINT and ID were significantly lower in the WB tolerant clone (CCN-51) in all treatments.

In SIC-876, sucrose and salicylic acid (SA) caused significant decrease ($p < 0.05$) of SINT and ID. While in CCN-51 there was a significant decrease of both variables only in seedlings treated with SA.

4.3.2. Macro and micronutrients mineral

In general, no significant reductions ($p < 0.05$) in the leaf concentration of mineral nutrients between treatments for both genotypes were found (Figure 3). However, in SIC-876 (Figure 3A), K values were significantly ($p < 0.05$) lower in plants treated with glucose. Also, Ca and Mg concentrations were significantly ($p < 0.05$) lower in plants treated with sucrose. In CCN-51 no significant differences were observed in the mineral macronutrients content at the leaf level (Figure 3B), except

for Ca concentrations that were significantly ($p < 0.05$) lower in plants treated with glucose and SA.

Additionally, significantly greater values of N, Ca and Mg were shown by SIC-876 compared to CCN-51. On the other hand, the reverse was determined for Mg. Phosphorous concentration was statistically similar between genotypes and that of K varied with the applied elicitors for SIC-876 (Figure 3).

Regarding the concentration of leaf mineral micronutrients no significant differences were observed for Zn and Cu in SIC-876 (Figure 4A) and CCN-51 (Figure 4B). However, CCN-51 had higher Zn concentration in all treatments than SIC-876. The Cu content was similar for both genotypes. However, significant differences were observed ($p < 0.05$) for Fe and Mn. Iron levels were significantly ($p < 0.05$) lower in plants treated with SA in SIC-876; in CCN-51 the concentrations of Fe and Mn were significantly ($p < 0.05$) lower in plants treated with sucrose and in control plants. Furthermore, the application of glucose and SA caused an increased Fe uptake in CCN-51 with respect to SIC-876 (Figure 4). Regarding Mn leaf concentrations, the values were significantly ($p < 0.05$) lower in plants of SIC-876 treated with sucrose and SA. In CCN-51, Mn values were significantly ($p < 0.05$) lower in plants treated with glucose and SA. The comparison between graphs (A and B) in Figure 4 shows Mn content differences between the two genotypes in relation to glucose and SA. This difference suggests different behaviors in the absorption of these nutrients depending on the elicitor applied.

4.3.3. Leaf gas exchange

Overall there were no significant differences shown in photosynthetic rates (*A*), either among elicitors as in genotypes (Figure 5A). However, *A* values were

significantly higher ($p < 0.05$) in glucose treated plants of CCN-51 compared to SIC-876. For stomatal conductance to water vapor (*gs*) (Figure 5B), significantly ($p < 0.05$) higher values were observed in the control treatment of both genotypes and in CCN-51 seedlings treated with glucose. It was observed a significant correlation between the behavior of *A* and *gs* in both genotypes in all treatments, except for SA (Figure 5).

In general, significant differences ($p < 0.05$) were found among genotypes with respect to transpiration rate (*E*) (Figure 6A) and instantaneous water use efficiency (*A*/*E*) (Figure 6B), except for plants treated with SA.

Both clones showed values of *A*/*E* significantly ($p < 0.05$) higher in plants treated with SA. On the other hand, CCN-51 did not show significant differences among seedlings treated with sucrose, glucose and SA; however, the values of *A*/*E* were significantly lower in control plants. Also, CCN-51 showed significantly higher *A*/*E* values when compared with SIC-876 for the sucrose and glucose treatments (Figure 6).

4.3.4. Chlorophyll fluorescence

Variables of fluorescence emission, in general, showed significant changes ($p < 0.05$) among elicitors and genotypes. However, there was no significant difference ($p < 0.05$) among genotypes for initial fluorescence (F_0) (Figure 7A) in the control treatment and in plants treated with sucrose. Moreover, the behavior of these genotypes in relation to the application of glucose and SA was opposite. F_0 was significantly higher for SIC-876 in the glucose treatment and lower in the SA treatment, contrary to CCN-51.

Significantly ($p < 0.05$) higher maximal fluorescence (F_m), values were observed in CCN-51 in all treatments. In CCN-51 there were no significant differences among treatments (Figure 7B). Similar behavior was found for the maximum quantum yield (F_v/F_m) values of photosystem 2 in this genotype. For SIC-876, we found significant differences ($p < 0.05$) for F_m and F_v/F_m in seedlings treated with glucose (Figure 7C).

4.4. Discussion

4.4.1. Symptom and disease index

Salicylic acid plays a central role as signal transducer, involved in plant defense against microbial attacks (Mauch et al., 1988). Exogenous application of SA, which effectively induced gene expression of proteins involved in the induction of systemic acquired resistance (SAR), led to the investigation of the role of endogenous SA in disease resistance (Chet, 1993). It is known that SA promotes SAR and the accumulation of PR-proteins in many plant species (Kessmann et al., 1994). Cacao seedlings treated with SA of both genotypes (SIC-876 and CCN-51) had lower proportions of plants with WB and lower DI (Figure 2). In the susceptible genotype SIC-876, SA and sucrose were statistically similar. Sugars are cellular nutrients which act as modulators of genes in yeast, plants and animals. In plants play an important role as nutrient and signal transducer. Both glucose and sucrose are essential as part of regulatory molecules that control the expression of genes related to the metabolism of plant resistance to stresses, growth and development (Rolland et al., 2006).

Salicylic acid is an endogenous growth regulator of phenolic nature that participates in the modulation of physiological processes in plants. It plays an

important role in the induction of thermogenesis and flowering, controls the absorption in roots and stomatal conductance, participates in the regulation of gene expression and regulatory gravitropism and inhibition of fruit ripening (Raskin, 1992; Morris et al, 2000; Medvedev; Markova, 1991, Srivastava; Dwivedi, 2000).

Recently, SA has drawn attention due to its ability to induce resistance in plants against different pathogens through the expression of proteins related to pathogenesis (Metraux, 2001).

The application of potassium chloride, glucose, sucrose and SA in treated cacao plants showed an effective action in the induction of resistance to WB (Vieira and Valle, 2006). Sucrose applications on *Lupinus luteus* L. cv. Polo provided increased defense responses against *Fusarium* sp. (Morkunas et al., 2005). These results show that soluble sugars are involved in mechanisms of resistance (Trouvelot, 2014).

4.4.2. Mineral macro and micronutrients

In general, the elicitor used affected the concentrations of N, P, K and Mg in both genotypes, except for glucose (K) and sucrose (Mg) in SIC-876. Therefore, the absorption of these macronutrients is not affected by the applied elicitors. The highest concentration of N, Ca and Mg in SIC-876 in relation to CCN-51 (Figure 3) may be due by differences in the requirements of these nutrients by each of the studied genotypes. The low concentration values of Ca and Mn in both genotypes coincide with more effective elicitors (sucrose and SA) in SIC-876 and AS in CCN-51. Ca and Mn have low mobility in the plant. In the case of Mn, this is due to the restricted flow of Mn in the phloem or by solubility problem, even though, this issue needs to be clarified (Page et al., 2006). In addition, Mn is a cofactor of superoxide

dismutase, therefore, participates in plant defense against oxidative stress produced by high levels of activated oxygen and free radical forms (reactive oxygen species, ROS), which are harmful to plants (Ducic; Polle, 2005; Hänsch; Mendel, 2009).

Physiologically, the use of elicitors in order to induce resistance often requires a cost for plant adaptation, it may have a negative effect on their development and production when plants are not infected (Heil and Bostock, 2002). However, induced resistance under natural conditions represents costs only in the presence of the pathogen (Heil and Baldwin, 2002).

4.4.3. Leaf gas exchange

Values of *gs* are related to stomatal density, control of water loss and CO_2 assimilation by plants for maintenance of photosynthetic rates (Taiz; Zeiger, 2013), having a close relationship in the control of *E.* Changes in *E* rates cause changes in several physiological processes, such as temperature and leaf water potential (Farquhar; Sharkey, 1982, Farquhar; Wong, 1984). In general, the values of *A*, *gs* and *E* observed in this study (Figure 4 and Figure 5A), were higher in control and glucose treated seedlings. All resource allocation for defense can be considered as a general cost, but this cost is divided between constitutive and inducible defenses (Coley et al., 1985). Moreover, maintaining higher photosynthetic rates under stresses have a high energy cost (Hikosaka; Terashima, 1995).

4.4.4. Chlorophyll fluorescence

Differences in the F_m values may show variations in the properties of electron acceptors of PS2 caused by conformational changes induced by stress in the main

constituent of the protein complex, the D_1 protein, which forms PS2 (Bulkhov et al., 1999). The variable fluorescence (F_V) is the increment of fluorescence from F_0 to F_m. Shows the capacity of PS2 to perform photochemical reduction (reduced QA) (Baker, 2008). F_V/F_m is an estimative of maximum quantum efficiency of PS2 photochemical activity when all reaction centers are open (Baker, Rosenquist, 2004). This ratio has been constantly used to detect disturbances in the photosynthetic system caused by biotic and abiotic stresses, since its decrease may indicate inhibition of photochemical activity (Konrad et al., 2005). Generally, the chlorophyll fluorescence parameters were not influence by the elicitors, the differences observed occurred among genotypes. The highest values of F_m and F_V/F_m were observed in CCN-51 (Figure 7).

4.5. Conclusions

Sucrose, glucose and salicylic acid have an induction effect for resistance to witches' broom disease in cacao.

Salicylic acid was the most efficient elicitor for induction of resistance to WB in both genotypes of *T. cacao*, since promoted decrease of brooms and disease severity on both genotypes, however, the sucrose was also effective in that reduction in SIC-876, the more susceptible material.

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Figure Legends

Figure 1 - Progression of witches' broom (WB) symptoms in cacao seedlings under greenhouse conditions. DAI = days after inoculation, N.I. = Not inoculated leaf , Inf. = Infected leaf. (Adapted from Teixeira et al., 2014

Figure 2 - Mean values of symptoms (A) and disease index (ID) (B) in cacao genotypes at 60 days after inoculation with *Moniliophthora perniciosa* spores. Capital letters indicate comparison among treatments for SIC-876; lowercase letters indicate comparison among treatments for CCN-51 by Tukey test ($p < 0.05$). Asterisks indicate comparison between genotypes by the t test ($p < 0.05$) within each treatment. Means ± SD; n = 4.

Figure 3 - Leaf mineral macronutrient concentrations of *T. cacao* genotypes SIC-876 (A) and CCN-51 (B) treated with chemical elicitors 60 DAI with *M. perniciosa* spores. Letters indicate comparison between treatment means within macronutrient by Tukey test ($p < 0.05$). Means ± SD; n = 4.

Figure 4 - Leaf mineral micronutrient concentrations of *T. cacao* genotypes SIC-876 (A) and CCN-51 (B) treated with chemical elicitors 60 DAI with *M. perniciosa* spores. Letters indicate comparison between treatment means within micronutrient by Tukey test ($p < 0.05$). Means ± SD; n = 4.

Figure 5 - Net photosynthetic rate (*A*) (A) and stomatal conductance to water vapor (*gs*) (B) of *T. cacao* genotypes treated with chemical elicitors and inoculated with *M. perniciosa*. Measurements were made at 60 DAI. Capital letters indicate comparison among elicitors by Tukey test ($p < 0.05$); lower case letters indicate comparison between genotypes within elicitor by Student's t test ($p < 0,05$). Means ± SD; n = 6.

Figure 6 - Leaf transpiration rate (*E*) (A) and instantaneous water use efficiency (*A*/*E*) (B) of *T. cacao* genotypes treated with chemical elicitors and inoculated with *M. perniciosa*. Measurements were made at 60 DAI. Capital letters indicate comparison among elicitors by Tukey test ($p < 0.05$); lower case letters indicate comparison between genotypes within elicitor by Student's t test ($p < 0,05$). Means ± SD; n = 6.

Figure 7 - Initial fluorescence (F_0) (A), maximal fluorescence (F_m) (B and maximum quantum efficiency of photosystem 2 ((F_v/F_m) (C) of *T. cacao* genotypes treated with chemical elicitors and inoculated with *M. perniciosa*. Measurements were made at 60 DAI. Capital letters indicate comparison among elicitors by Tukey test ($p < 0.05$); lower case letters indicate comparison between genotypes within elicitor by Student's t test ($p < 0,05$). Means ± SD; n = 6.

Figure 1

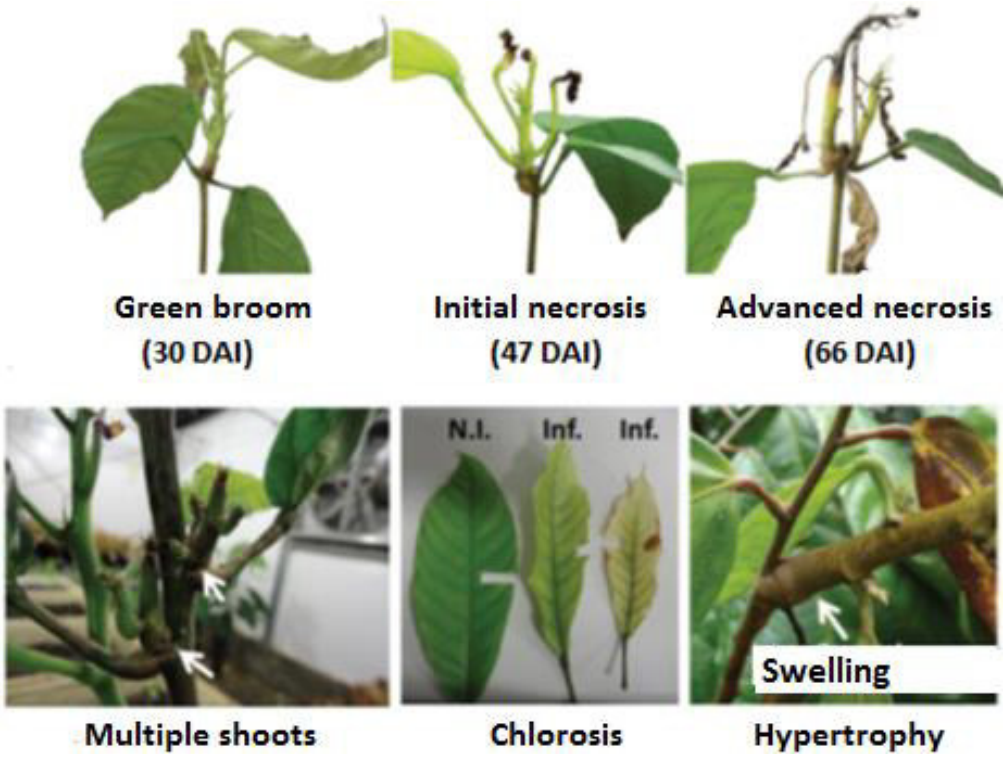

Green broom
(30 DAI)
Initial necrosis
(47 DAI)
Advanced necrosis
(66 DAI)
N.I.
Inf.
Inf.
Swelling
Multiple shoots
Chlorosis
Hypertrophy

Figure 2

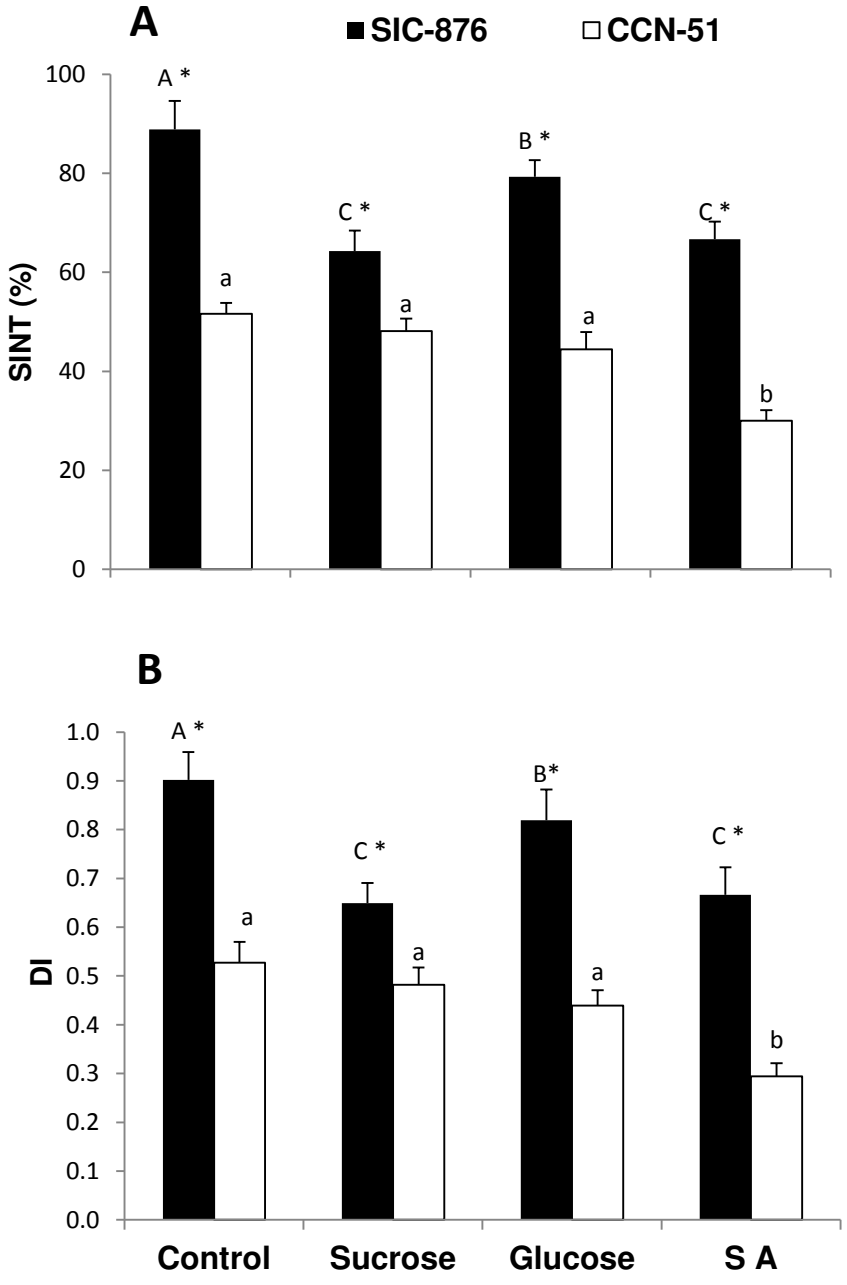

A
SIC-876
CCN-51
SINT (%)
100
80
60
40
20
0
A *
a
C *
a
B *
a
C *
b
B
DI
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A *
a
C *
a
B*
a
C *
b
Control
Sucrose
Glucose
S A

Figure 3

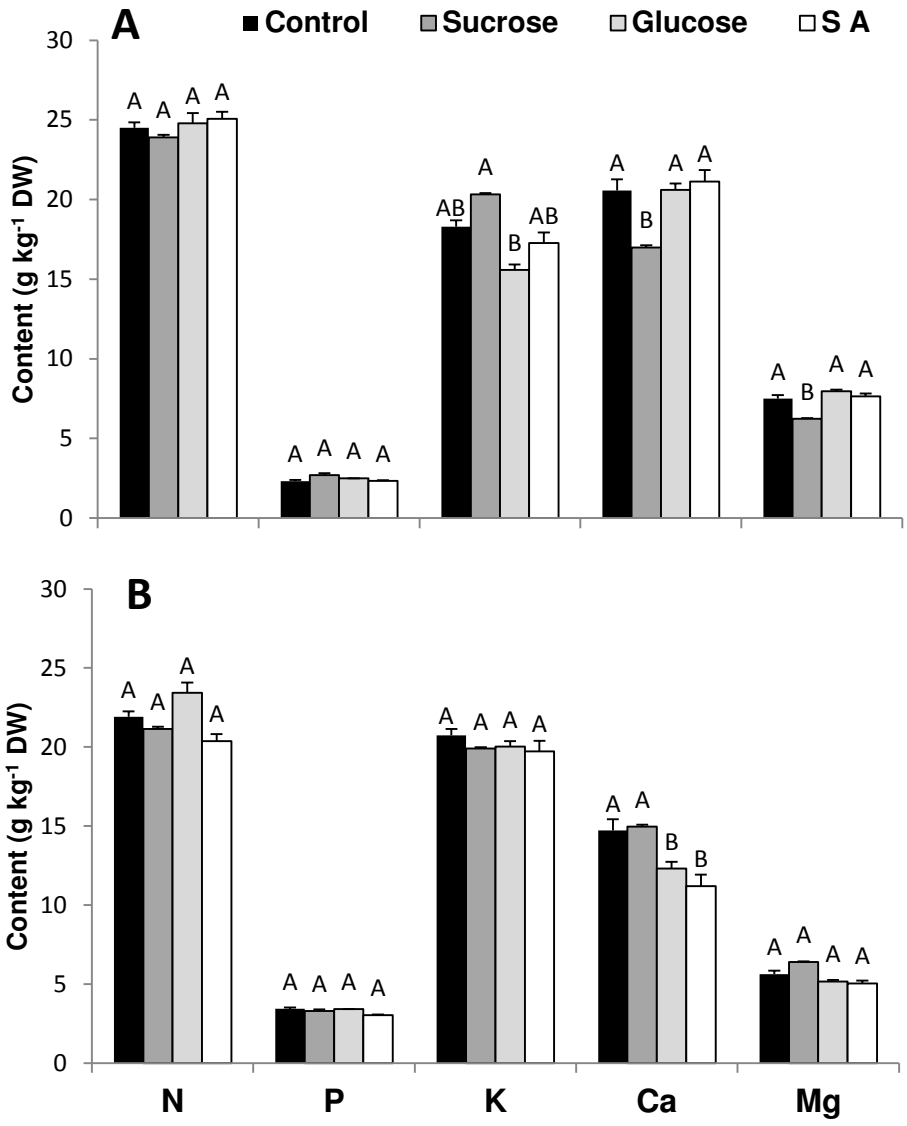

A
Control
Sucrose
Glucose
S A
Content (g kg-1 DW)
30
25
20
15
10
5
0
A A A A
A A A A
AB A B AB
A B A A
A B A A
B
Content (g kg-1 DW)
A A A A
A A A A
A A B B
A A A A
N
P
K
Ca
Mg

Figure 4

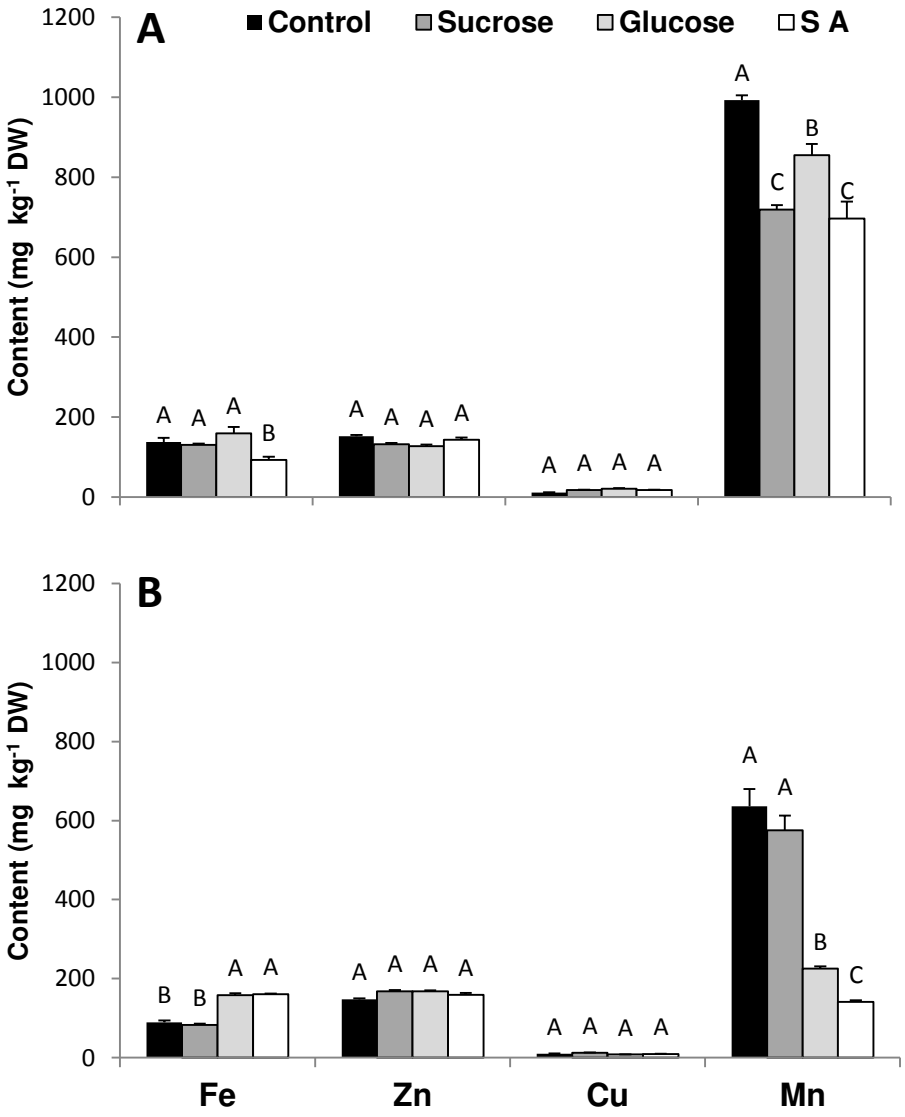

A
Control
Sucrose
Glucose
S A
Content (mg kg-1 DW)
1200
1000
800
600
400
200
0
A A A B
A A A A
A A A A
A C B C
B
B B A A
A A A A
A A A A
A A B C
Fe
Zn
Cu
Mn

Figure 5

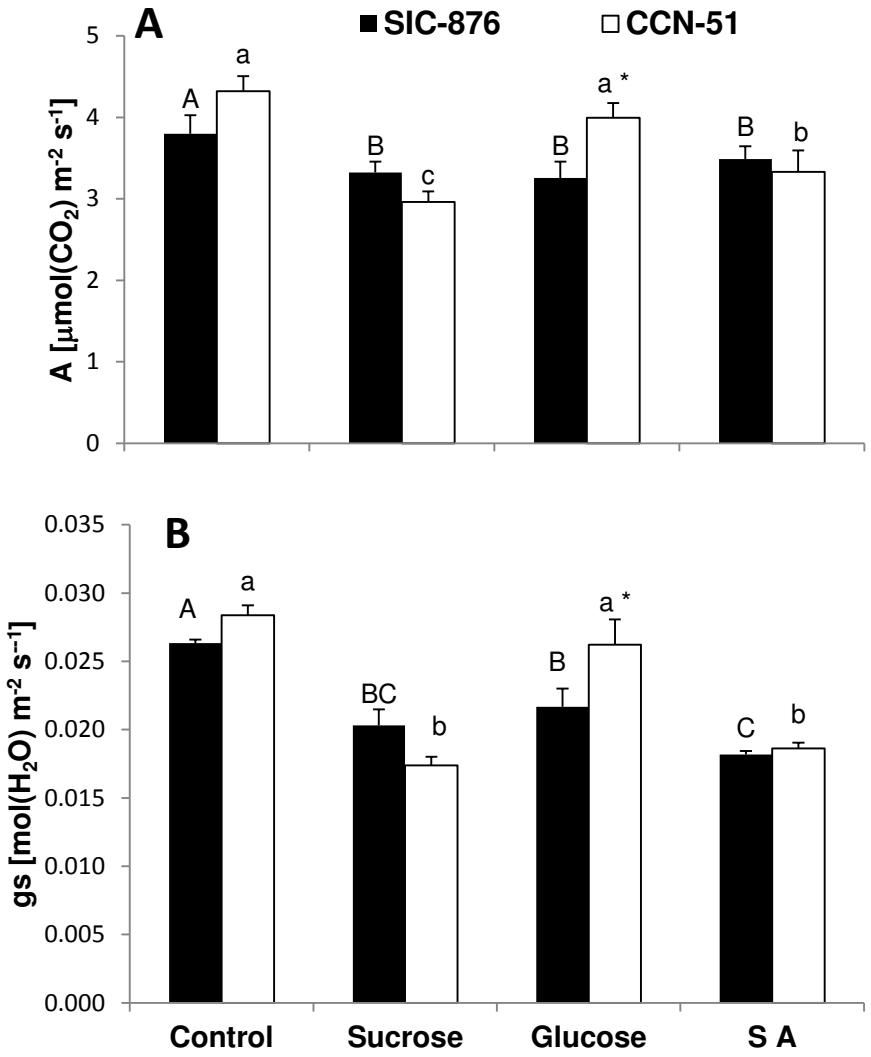

A
SIC-876
CCN-51
A [µmol(CO2) m-2 s-1]
5
4
3
2
1
0
A
a
B
c
B
a *
B
b
B
gs [mol(H2O) m-2 s-1]
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
A
a
BC
b
B
a *
C
b
Control
Sucrose
Glucose
S A

Figure 6

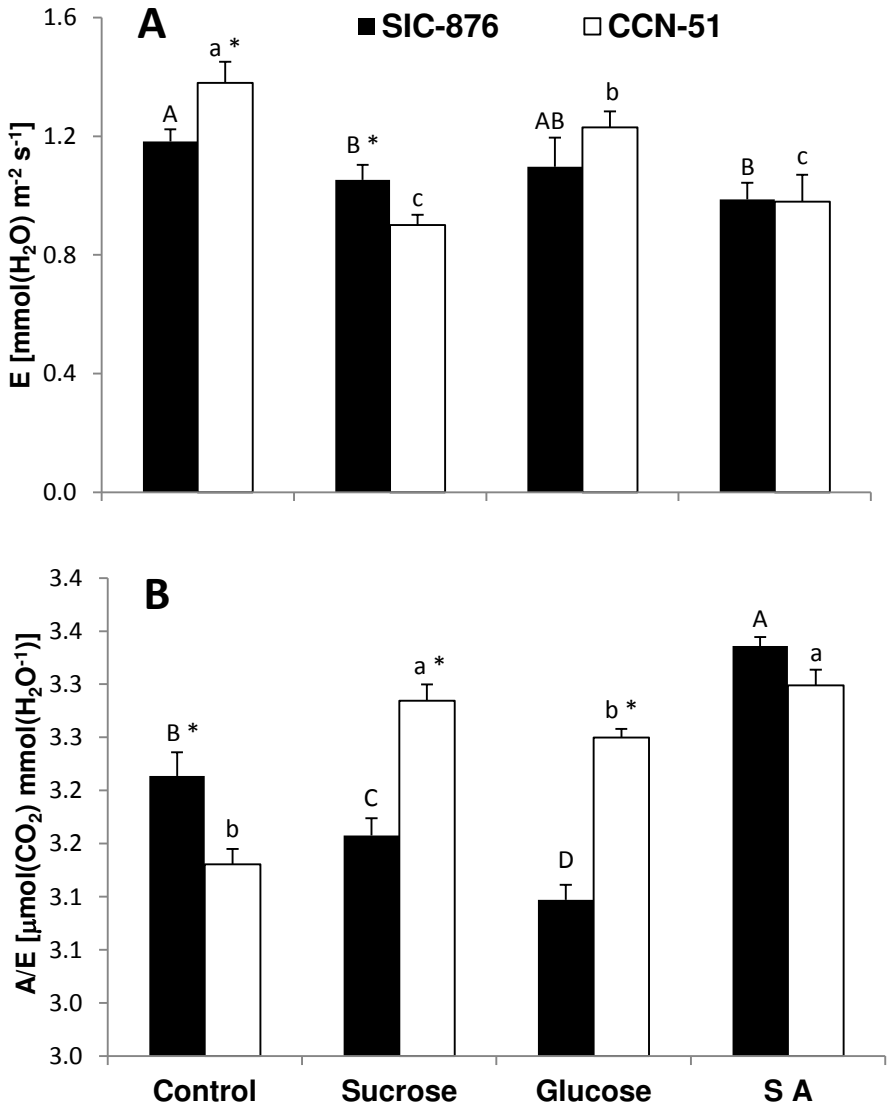
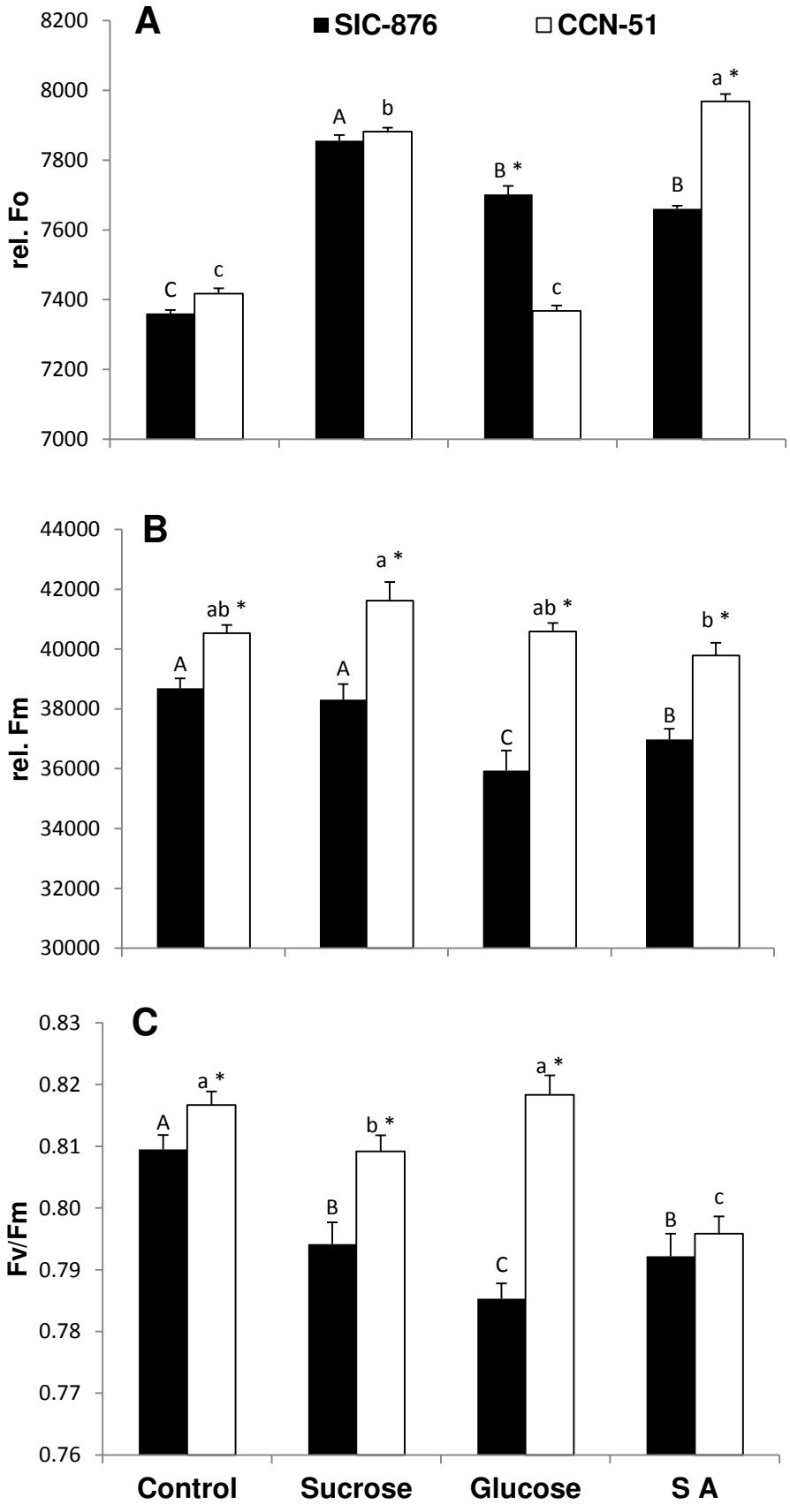

A
SIC-876
CCN-51
E [mmol(H2O) m-2 s-1]
1.6
1.2
0.8
0.4
0.0
a *
A
B *
c
b
AB
B
c
B
A/E [µmol(CO2) mmol(H2O-1)]
3.4
3.4
3.3
3.3
3.2
3.2
3.1
3.1
3.0
3.0
B *
b
a *
C
D
b *
A
a
Control
Sucrose
Glucose
S A

Figure 7


A
SIC-876
CCN-51
rel. Fo
8200
8000
7800
7600
7400
7200
7000
C
c
A
b
B *
c
B
a *
B
rel. Fm
44000
42000
40000
38000
36000
34000
32000
30000
A
ab *
A
a *
C
ab *
B
b *
C
Fv/Fm
0.83
0.82
0.81
0.80
0.79
0.78
0.77
0.76
A
a *
B
b *
C
a *
B
c
Control
Sucrose
Glucose
S A

5. CONCLUSÕES GERAIS

Houve, no tratamento controle, a confirmação da intolerância do genótipo de *T. cacao* SIC-876 e da tolerância do genótipo CCN-51 ao *M. perniciosa*.

As estratégias de controle da doença vassoura-de-bruxa, avaliadas por meio da substituição da parte aérea pela técnica de enxertia de topo e da indução de resitência por meio de elicitores químicos, se mostraram promissoras no convívio com a doença.

As respostas fisiológicas das interações entre enxerto (CCN-51, tolerante) e porta-enxerto (SIC-876, intolerante ao *M. perniciosa*) de *T. cacao*, demonstraram que a técnica de enxertia poderá ser uma ferramenta importante no controle da doença vassoura-de-bruxa.

O porta-enxerto SIC-876 proporcionou maior tolerância do enxerto CCN-51 à vassoura-de-bruxa, devido, principalmente, ao aumento na eficiência do uso instantâneo de água, associado à maior atividade das enzimas peroxidase do ascorbato (APX) e do guaiacol (GPX), envolvidas no metabolismo antioxidativo.

O elemento metálico manganês está associado aos efeitos sinergísticos dos elicitores químicos glicose, sacarose e ácido salicílico no controle da vassoura-de-bruxa do cacaueiro.

O ácido salicílico foi o único indutor efetivo para o genótipo tolerante CCN-51 em relação à diminuição do sintoma e à severidade da vassoura-de-bruxa. A aplicação dos elicitores químicos sacarose, glicose e ácido salicílico via foliar promoveram diminuição dos sintomas da doença no genótipo intolerante SIC-876.

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